THE SOCIETY FOR APPLIED BACTERIOLOGY
TECHNICAL SERIES NO. 2

IDENTIFICATION METHODS FOR MICROBIOLOGISTS

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PART B

ACADEMIC PRESS · LONDON · NEW YORK

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ACADEMIC PRESS INC. (LONDON) LTD
BERKELEY SQUARE HOUSE
BERKELEY SQUARE
LONDON, W.1

U.S. Edition published by
ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK, NEW YORK 10003

Library of Congress Catalog Card Number: 66–20871

Printed in Great Britain by
W. & J. Mackay & Co Ltd, Chatham, Kent

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Preface

This volume contains material from the Autumn Demonstration Meeting of the Society for Applied Bacteriology which was held on the 26th October 1966 at the Sir John Atkins Laboratories, Queen Elizabeth College, University of London. Some of those groups known to present identification difficulties and whose elucidation was thought to be valuable to members of the Society were selected for demonstration. Demonstrators expert with a group of organisms were given a pure culture known to belong to that group, and asked what methods and techniques they would use in order to identify it. Papers based on their demonstrations were invited, therefore each paper is an entity not a part of some overall theoretical scheme.

It is sad to have to record the sudden and untimely death of Mr B. M. Gibbs when the editorial arrangements for this volume had been partially completed. B. M. Gibbs did much to initiate the demonstration meetings of the Society on which this present series of books is based. His death is a great loss to the Society and is felt deeply by all who knew him.

The editors would like to express appreciation to Dr F. A. Skinner and to Mr A. Harry Walters for their valuable help and advice given to complete this volume.

Our particular thanks go to Professor S. J. Pirt, Dr G. Anagnostopoulos and other members of the staff of the Microbiology Department of Queen Elizabeth College for their help with the laboratory arrangements for the demonstrations.

B. M. Gibbs
D. A. Shapton

February 1968

Contents

Methods for Identifying Acetic Acid Bacteria

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Although vinegar is used almost universally for culinary purposes, knowledge of the organisms participating in its manufacture and of the related species is not so widespread. In the undomesticated form these bacteria are found as contaminants of various fermented liquors and also in association with the raw materials, particularly if these happen to be fruit. In recent years the acetic acid bacteria have been divided into two genera. Differences in flagella pattern between the two groups was first observed by Leifson (1954), who suggested that the peritrichously flagellated organisms and those non-motile ones of similar biochemical constitution should retain the name *Acetobacter*, while the polarly flagellated ones should be called *Acetomonas*. Further support was given to this by Shimwell and Carr (1959), who formally proposed the creation and recognition of the genus *Acetomonas*. Table 1 shows some of the differences between the two genera. It is interesting to note that only members of the genus *Acetobacter* constitute the working organisms of a vinegar generator. Although *Acetomonas* species are able to produce acetic acid, they do so rather inefficiently and cannot be used for vinegar manufacture. They are more likely to be found in habitats containing sugars (fruit juices, rotting fruit, beer wort), which they utilize readily.

It has been suggested by De Ley (1961) that a more appropriate name for *Acetomonas* would be *Gluconobacter*. This name was first coined by Asai (1934, 1935) and applied to a mixed group of bacteria that contained: (*a*) *Acetobacter* species; (*b*) organisms that would now be called *Acetomonas* species; (*c*) a group of acid-tolerant organisms that in some respects resembled acetomonads, but were unable to oxidize ethanol to acetic acid. Since De Ley's (1961) suggestion was based upon the incorrect identification of at least one organism (Hodgkiss, Shimwell and Carr, 1962; Kimmitt and Williams, 1963), it would seem more appropriate to use the less equivocal name *Acetomonas* for polarly flagellated acetic acid bacteria. The name *Gluconobacter*, if it is to be used at all, might be applied to organisms of group (*c*), as suggested by Lott and Carr (1964).

TABLE 1. Properties of *Acetobacter* and *Acetomonas*

	Acetobacter	*Acetomonas*	Author
Ethanol oxidized to $CO_2 + H_2O$	+	—	
Lactate oxidized to carbonate	+	—	Frateur (1950)
Nutrition type	Lactophilic	Glycophilic	Brown and Rainbow (1956)
Oxidize various amino acids	+	—	Joubert, Bayens and De Ley (1961)
Citric acid cycle	+	—	Cheldelin (1960)*
Flagella (if motile)	Peritrichous	Polar	Leifson (1954)
Serology	Hetrogeneous	Homogeneous	McIntosh (1962)
Infrared spectra	Differ from strain to strain but all distinguishable from *Acetomonas*	Differ from strain to strain but all distinguishable from *Acetobacter*	Scopes (1962)

* Cheldelin uses the name *Acetobacter suboxydans* in this publication. This and *A. melanogenum* were the names most commonly applied to the majority of organisms that now constitute the genus *Acetomonas*.

Identification at Generic Level

Although Table 1 shows a number of characteristics that might be tested to decide whether the organisms under examination are *Acetobacter* or *Acetomonas*, in practice the only test required is the first one listed. This will show whether they produce acid from ethanol and, secondly, whether they subsequently destroy the acid. Two media are available for this test, the first having been devised by Frateur (1950). The composition is as follows: Difco yeast extract, 3%; $CaCO_3$ (as finely divided as possible), 2%; agar, 2%. This and all other media subsequently described are sterilized by autoclaving at 121° unless otherwise stated. When molten, 2 ml of 15% sterile filtered ethanol/13 ml are added, the mixture shaken to disperse the chalk and poured into plates. About 5 cultures may then be spread on the surface in circular areas about 1 cm in diameter. Acid production is then indicated by the formation of a cleared area around the bacterial growth. *Acetomonas* cultures remain at this stage, whereas *Acetobacter*

species begin to redeposit chalk from the edge of the colony outwards in the cleared area. This was described by Frateur (1950) as "irisation" in reference to the characteristic sheen that redeposited chalk has as it catches the light. This method is slow and irisation difficult to detect. An easier method is to use the following medium: Difco yeast extract, 3%; agar, 2%; bromo-creosol green (added at the rate of 1 ml of a 2·2% solution/l). This is most conveniently dispensed as 6·5 ml aliquots, in McCartney bottles. When molten, 1 ml of 15% sterile filtered ethanol is added to give a final concentration of 2%. Bottles can be sloped, inoculated, and should be incubated with the caps loosened at 28°. *Acetomonas* species will produce acid and change the indicator from an initial bluish-green to yellow. No further change takes place irrespective of the length of incubation. *Acetobacter* species also effect the same colour change to yellow and then, as the acid is oxidized, the colour reverts to bluish-green. Strains vary in the time taken to bring about these changes so that a specific period of incubation cannot be given. Some strains of *Acetobacter xylinum* are particularly slow in bringing about the second phase of oxidation, and prolonged incubation may be necessary for completion of this change.

A confirmatory test may be done by growing the organisms on the following medium: Difco yeast extract, 2%; calcium lactate, 2%; agar, 2%. If inoculations are done as for the ethanol chalk plates, the growth of *Acetobacter* species rapidly becomes surrounded by a halo of precipitated calcium carbonate. In contrast, the growth of *Acetomonas* species is very thin and produces no white precipitate in the medium.

Should it be necessary to stain the organisms to ascertain their flagella pattern several points of technique must be observed. Acetic acid bacteria show their greatest motility after an incubation of about 18 h at a maximum temperature of 25°. Flagella become detached very easily, so that considerable delicacy is required in suspending these organisms. For the staining method and further details of how to treat these organisms Shimwell (1959*a*) should be consulted.

Identification at Specific Level

Acetomonas

Shimwell and Carr (1959) have suggested that this genus should contain only a single species called *Acetomonas oxydans* (Henneberg) Shimwell and Carr. While there are many variants of this species, such as the technologically important producers of ropy beer (formerly known as *Acetobacter viscosus* and *Acetobacter capsulatum*), this characteristic is not considered sufficiently important to warrant specific rank. The characteristics of *Acetomonas oxydans* are listed in Table 2.

TABLE 2. Species of acetic acid bacteria

GENUS	Acetobacter								Acetomonas
SPECIES	*aceti*	*xylinum*	*mesoxydans*	*lovaniense*	*rancens*	*ascendens*	*peroxydans*	*paradoxum*	*oxydans*
Overoxidation of ethanol	+	+	+	+	+	+	+	+	—
Catalase	+	+	+	+	+	+	—	—	+
Growth in Hoyer's medium	+	—	—	+	—	—	+	—	—
Acid from glucose	+	+	+	+	+	—	—	—	+
Dihydroxyacetone from glycerol	+	+	+	—	—	—	—	—	+
Production of cellulose	—	+	—	—	—	—	—	—	—
Brown pigment	—	—	—	—	—	—	—	—	+

Acetobacter

One of the first references to the instability of *Acetobacter* species is that of Schramm and Hestrin (1954), who showed that *A. xylinum* would, under submerged, aerated culture conditions, lose its ability to produce extracellular cellulose. It was Shimwell (1957*a*) who pointed out that by the criteria shown in Table 2 this species had become *A. mesoxydans*. He had shown in the previous year (Shimwell, 1956) that a strain of *A. mesoxydans* used in small pilot-scale sterilizable generators was able to change with great facility either to *A. xylinum* by gain of cellulose production or to *A. rancens* by loss of the ability to produce dihydroxyacetone. Since then many other examples of this type of mutability amongst these organisms has been recorded (Shimwell, 1959*b*; Shimwell and Carr, 1960; Carr and Shimwell, 1961). This raises the whole question of whether *Acetobacter* species as listed in Table 2 are, in fact, genuine species or whether they cover the whole mutational range of a single species. One noteworthy point is that *A. aceti*, *A. lovaniense* and *A. peroxydans* (all Hoyer positive) show considerable variation of colony form within a single species. These differing colony forms, however, are quite uniform in respect of the biochemical characteristics listed in Table 2. The remaining *Acetobacter* species also exhibit diverse colony forms within a single species, and some can be shown to differ biochemically from the parent. Indeed, it is sometimes possible to pick off two apparently identical colonies from the same culture and to find that they differ in respect of the biochemical tests of Table 2. It must be emphasized that, having identified a culture by means of the subsequent tests, there is no guarantee that the same answer will be obtained after a few months' subculture. It is, therefore, recommended that acetic acid bacteria should be identified as soon as possible after isolation and maintained as freeze-dried cultures.

Application of the specific tests

Catalase production

A number of media can be used for this test, providing they are not too acid. It is performed in the conventional way by the application of hydrogen peroxide to growth on a solid medium and observing evolution of gas bubbles. The most convenient medium for this purpose is the one used to detect acid from glucose to be described later.

Growth in Hoyer's medium

The composition of Hoyer's medium is as follows: $(NH_4)_2SO_4$, 0·1%; K_2HPO_4, 0·01%; KH_2PO_4, 0·09%; $MgSO_4.7H_2O$, 0·025%; $FeCl_3.6H_2O$,

0·002%; ethanol, 3%. The most convenient method of preparation and use is to dissolve the salts in $\frac{4}{5}$ of the final volume of distilled water, dispense as 4 ml aliquots into test tubes and autoclave. Ethanol may then be added as a 15% v/v sterile filtered solution at the rate of 1 ml per tube. Care must be taken when inoculating this medium to avoid transference of excess organic material. Because of the small initial inoculum and relatively unfavourable conditions, growth will be slow and may take up to 14 days to develop.

Acid from glucose

A suitable medium for this test is as follows: Difco yeast extract, 3%; glucose, 10%; $CaCO_3$, 3%; agar, 2%. Several organisms may be spread on each plate; a positive reaction is indicated by the development of a cleared area around the bacterial growth. Those giving a negative reaction will often grow, but produce no clearing. After observing these plates for acid production, they may then be used to detect catalase.

Dihydroxyacetone from glycerol

A simple medium with the following composition is used: Difco yeast extract, 3%; glycerol, 3% v/v; agar, 2%. Dihydroxyacetone may be detected easily by flooding inoculated plates with Fehling's solution. The positives are rapidly surrounded by a halo of cuprous oxide while negatives remain unaltered. Inocula on these plates should be well spaced and incubation should not exceed 48 h. Incubation for a longer period or crowding the plates will allow powerful dihydroxyacetone producers to fill the plate with this substance, thus obscuring any negative organisms that might be present.

Production of cellulose

Almost any medium containing a simple sugar such as glucose is suitable for this test. Production of cellulose can be recognized by the presence of a yellowish-cream cartilaginous pellicle and a clear subnatant fluid. A confirmatory test may be performed by staining the pellicle with Lugol's iodine and applying 60% H_2SO_4. Threads of cellulose stain a bright blue.

Production of brown pigment

This may be observed on a number of media containing glucose and can be seen on the following: Difco yeast extract, 2%; glucose, 2%; $CaCO_3$, 2%; agar, 2%. This may be dispensed conveniently as slopes. Since the medium tends to brown, it is often helpful to grow organisms of unknown reaction with a known negative and positive for comparison. This browning is very common amongst *Acetomonas* strains and is preceded by the forma-

tion of 2,5-dioxogluconic acid. It is a diffusible pigment and cannot, therefore, be confused with the pink non-diffusible pigments which sometimes colour these organisms. Although not shown in Table 2, there are two strains of *A. aceti* that produce brown pigment. The same phenomenon can be produced with some *Acetomonas* strains and fructose as substrate (Carr, Coggins and Whiting, 1963). This being a 2-oxohexose is converted by some organisms to a 2,5-oxohexose, thus forming a suitable starting material for the browning reaction.

Other *Acetobacter* Species

There are a large number of strains of acetic acid bacteria mentioned in the literature that are not included in the present scheme. Some of these have been lost, others have been found to be synonyms of other species. There are, however, two species that should be mentioned. The first of these is *Acetobacter pasteurianum*, unusual amongst bacteria in that it produces starch. During laboratory cultivation it loses the ability to produce starch and when devoid of this characteristic is indistinguishable from *A. rancens*. This gradual loss of starch is a characteristic noted by many workers handling this organism and a well-illustrated explanation of its mechanism has been given by Shimwell (1957*b*).

The other organism that should be mentioned is one named *Acetobacter acidophilum* by Wiame, Harpigny and Dothey (1959), who isolated it from a trickling vinegar generator. The most unusual characteristic is its inability to grow above pH 4·3. As these authors admitted, this made the organism hard to handle and it would be difficult to apply the type of tests described in this paper. From their description, the organism is of low oxidative capacity, probably resembling a strain of *A. peroxydans* with a low pH optimum. There is reason to suppose that by adapting the tests recorded in Table 2 to a lower pH range it would be possible to establish this organism's relationship with other acetic acid bacteria. The fact that it has a low pH optimum is unusual, but not more so than the failure of several of these highly aerobic organisms to produce catalase.

Conclusion

The foregoing presents a scheme of identifying acetic acid bacteria which, it is hoped, will be of use to the working bacteriologist who is not generally interested in the minutae of taxonomy, but rather in the sort of organisms he is isolating from a particular source. It is not claimed even to be the best scheme, but it has the virtues of simplicity in use and interpretation. It is also a reminder that living organisms are not stable: but that some are more stable than others.

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Identification of Human Vibrios and Allied Organisms

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From the early days of bacteriology a wide variety of Gram-negative rods have been included in the genus *Vibrio* almost solely because their long axis has shown some curvature. The heterogeneity of so-called vibrios is clearly shown by studies on their DNA base compositions as summarized by Hill (1966). The advent in recent years of these and other tests for the precise identification and classification of bacteria has helped to reduce the chaos within this genus (Davis and Park, 1962; Sebald and Véron, 1963). Further, recent taxonomic studies on other oxidase-positive, fermentative, polarly flagellate, Gram-negative rods (Ewing, Hugh and Johnson, 1961; Habs and Schubert, 1962; Eddy and Carpenter, 1964) have indicated the relationship of the three groups of organisms conforming to this description, viz. *Vibrio*, *Aeromonas*, *Plesiomonas*. Indeed, Eddy and Carpenter proposed that these three groups (or genera) might be placed in one family, possibly named Vibrionaceae, and a similar proposal was made by Veron (1965). It is the identification of organisms of importance in medical bacteriology that belong to this family, as defined here, which will be described in this chapter.

Nomenclature and Pathogenicity

Vibrio

Currently the term "cholera" includes the exclusively human disease caused not only by the so-called "classical" *Vibrio cholerae* but also by the El Tor vibrio, as the disease caused by either organism is clinically indistinguishable. There is much support for the opinion that the differences between these organisms are of infrasubspecific significance only and that they may be regarded as biotypes of one "species" (Feeley, 1965; Hugh, 1965*a*, *b*). In this chapter, therefore, the term "cholera vibrios" includes both the "classical" and El Tor biotypes and for convenience, without prejudice to

their ultimate designation, they are named *Vibrio cholerae* and *Vibrio* El Tor.

The term "non-cholera vibrio" (NCV) or "non-agglutinable" vibrio (NAG) includes other vibrios, mainly from human and water sources, which do not agglutinate in Polyvalent *Vibrio cholerae* 0 group 1 serum (McIntyre and Feeley, 1965). They do not cause cholera, but are sometimes associated with a diarrhoeal syndrome in man.

Vibrio parahaemolyticus is the name proposed by Sakazaki, Iwanami and Fukumi (1963) for the facultatively halophilic organism encountered mainly in Japan as a cause of acute food poisoning associated with the consumption of sea food and fish products. Previous names were *Pasteurella parahaemolytica* and "*Pseudomonas enteritis*".

Aeromonas

Aeromonas formicans is used here to indicate the biotype of *Aeromonas liquefaciens* (*hydrophila*, *punctata*) which is typically anaerogenic, Voges-Proskauer-negative and gluconate-negative. Its pathogenicity for man is unknown.

Plesiomonas

Plesiomonas shigelloides (Habs and Schubert, 1962) is the designation now given to the organism "Paracolon C27" first described by Ferguson and Henderson (1947) and successively named *Pseudomonas shigelloides* (Bader, 1954), *Pseudomonas michigani* (Sakazaki *et al.*, 1959) and *Aeromonas shigelloides* (Ewing, Hugh and Johnson, 1961). Some strains contain the antigen of *Shigella sonnei* Phase 1. Its pathogenicity for man and animals is unknown.

Methods

Biochemical tests and media

The temperature of incubation is 35° unless otherwise stated. In all media Evans's peptone has been substituted for other peptones described in the original formulae. If a culture is suspected as being *Vibrio parahaemolyticus*, the sodium chloride content of the media should be raised to 2–3%.

Oxidase test. The filter-paper technique of Kovacs (1956) is used and the reagent is 1% (w/v) *p*-aminodimethylaniline oxalate (Difco) in distilled water.

Oxidation/fermentation test. The Hugh and Leifson (1953) medium is used.

Decarboxylases. The method of Møller (1955) is used.

Fermentation tests. Carbohydrates 0·5% (w/v) in 1% (w/v) peptone water with Andrade indicator are used and the reactions observed daily.

Indole. 2% (w/v) peptone water cultures are tested after incubation overnight with the reagent of Kovacs (1928).
H_2S production. This is detected by lead acetate papers over nutrient broth.
Urease. The medium of Christensen (1946) is used.
Phenylpyruvic acid (PPA) test. The production of PPA from phenylalanine is detected by the method of Henriksen (1950), or in the medium of Shaw and Clarke (1955) or on phenylalanine agar (Report, 1958, Test 18).
Methyl red (MR) and Voges-Proskauer (VP). Buffered glucose phosphate broth cultures are tested after incubation for 3 days at 30°. The VP test of O'Meara (1931) is used. Alternatively the method of Barritt (1936) may be used after overnight incubation at 35°.
Citrate utilization. The medium of Simmons (1926) is used.
Gluconate test. The method of Carpenter (1961) is used and the medium is that of Shaw and Clarke (1955).
Growth in KCN. The method of Møller (1954) is used, but the medium is contained in bijou bottles with the caps very tightly screwed.
Gelatin liquefaction. Nutrient gelatin stab cultures are grown at room temperature and observed daily.
Nitrate reduction. Cultures in 0·1% (w/v) nitrate broth are tested after incubation for 5 days by the Griess-Ilosvay method (Wilson and Miles, 1964).

Other tests

Motility. A preparation made from a young broth culture is examined by the hanging-drop method.
Serology. The polyvalent *V. cholerae* 0 group 1 serum contains antibodies to both the Ogawa and Inaba subtypes of cholera vibrios.
Phage sensitivity. The group IV phage of Mukerjee (1961) is used at the routine test dilution by the technique described by Mukerjee (1963).
Chick cell haemagglutination. The method is that of Finkelstein and Mukerjee (1963) as modified by Barua and Mukerjee (1965). Growth from an agar slope is emulsified on a slide in a drop of a 2·5% suspension in saline of washed chicken red cells. The slide is rocked for about one minute. Clumping of the red cells indicates a positive test. Sheep, rabbit, human and horse cells can also be used, but not guinea-pig cells.
Polymyxin B sensitivity. The method is that of Gan and Tjia (1963) using a plate technique with a disc containing 50μg of polymyxin B. Alternatively, 15 μg/ml of polymyxin B may be incorporated in a nutrient agar plate.
Sheep cell haemolysis. For the most reproducible results the method of Feeley and Pittman (1963) is best. 0·5 ml of an overnight heart infusion broth culture is mixed in a tube with 0·5 ml of a 1% suspension in saline of washed sheep red cells. The mixture is incubated at 35° for 2 h and

then left at 4° overnight before being read. Adequate controls are very necessary.

Identification

Because of the public health implications, rapid bacteriological confirmation of cholera is imperative and in the field is based on as few tests as possible, particularly on slide agglutination in specific serum of the organism isolated. The tests given in this chapter are those used to confirm the preliminary identification (Carpenter, 1966), and for epidemiological purposes. Isolation techniques are omitted.

TABLE 1. Differential tests in the identification of vibrios and allied organisms

OXIDASE +

HUGH AND LEIFSON
Utilization of glucose

Fermentative — DECARBOXYLASES: *Vibrio*, *Aeromonas*, *Plesiomonas*

Oxidative — DECARBOXYLASES: *Pseudomonas*

	Vibrio	*Aeromonas*	*Plesiomonas*	*Pseudomonas*
Arginine	—	+	+	+
Lysine	+	—	+	No change
Ornithine	+	—	+	No change

Key to Tables 1–4

A or +	= acid and not gas production (glucose only) or positive reaction in other tests.
(+)	= acid production or positive reaction after incubation for 48 h or longer.
+/— or —/+	= reaction before the oblique is given by a majority of strains.
d	= different strains give different reactions.

Key distinguishing characters

Table 1 shows the major tests to differentiate the oxidase-positive, polarly flagellate rods. The positive oxidase text rapidly distinguishes these organisms from the oxidase-negative Enterobacteriaceae. The decarboxylase tests are of major value in separating vibrios from aeromonads as many of their other biochemical reactions are very similar.

TABLE 2. Typical biochemical reactions

	Vibrio cholerae Vibrio El Tor	Non-cholera vibrio	*Vibrio para-haemolyticus*	*Aeromonas formicans*	*Plesiomonas shigelloides*
Glucose	A	A	A	A	A
Lactose	(+)	(+)/—	—	d	(+)
Mannitol	+	+	+	+	—
Sucrose	+	d	—	+	—
Dulcitol	—	—	—	—	—
Salicin	—	—	—	d	+/—
Xylose	—	—	—	—	—
Arabinose	—	d	d	+/—	—
Mannose	+	d	+	+/—	—
Starch	+	+	+	+	—
Adonitol	—	—	—	—	—
Inositol	—	—	—	—	+
Indole	+/—	+/—	+	d	+
H_2S	(+)/—	(+)/—	(+)	—	—
Urease	—	—	—	—	—
PPA	—	—	—	—	—
MR	d	d	+	+	+
VP	d	d	—/+	—	—
Citrate	+/—	+/—	+	+/—	—
Malonate	—	—	—	—	—
Gluconate	d	d	—	—	—
KCN	d	d	+	+/—	—
Gelatin	+	+	+	+	—
Nitrate reduction	+	+	+	+	+

All are oxidase-positive, fermentative, polarly flagellate, Gram-negative rods. For decarboxylase reactions see Table 1.

Typical biochemical reactions

Table 2 gives the typical reactions in the standard set of tests used routinely in the authors' laboratory (Carpenter, Lapage and Steel, 1966), and are the reactions given by a majority of strains.

As the *Vibrio* and *Plesiomonas* genera are invariably anaerogenic, the anaerogenic biotype of *Aeromonas*, *Aeromonas formicans*, has been chosen for comparison, as it is the most likely to be confused with vibrios. However, strains intermediate between the typical aerogenic *Aeromonas liquefaciens* and the typical anaerogenic *Aeromonas formicans* are found which are aberrant in one or two characters.

The cholera red (nitroso-indole) reaction is omitted, as it is not now considered a specific test for cholera vibrios. The nitrate and indole tests are best done separately.

Inositol fermentation is a most useful test in rapidly distinguishing *Plesiomonas* from *Vibrio* and *Aeromonas*.

The detailed lipolytic and proteolytic tests used by Eddy (1962) and Eddy and Carpenter (1964) are not done regularly. The test for sensitivity to the vibriostatic compound 0/129 (Shewan, Hodgkiss and Liston, 1954) and the "string" test of Smith (1958) using a sodium deoxycholate solution, which may both be useful in differentiating vibrios from aeromonads, are not done routinely.

Subdivision of vibrios

Figure 1 and Tables 3 and 4 show how vibrios may be subdivided.

Serology. Both *Vibrio cholerae* and the El Tor vibrio belong to 0 group 1 of Gardner and Venkatraman (1935) and cannot be distinguished serologically from each other. The cholera vibrios can be further divided for epidemiological purposes into two main subserotypes, Ogawa and Inaba, by the use of specific absorbed sera.

Heiberg biogroups. Heiberg (1936) described six groups based on the fermentation patterns in mannose, sucrose and arabinose. Two further groups were added by Smith and Goodner (1965). The reactions are given in Table 3.

All cholera vibrios belong to group (biogroup) I, as do some NCV's. The Heiberg tests are not diagnostic of the *Vibrio* genus and other tests must always be used in conjunction. Many other genera give similar Heiberg patterns. *Aeromonas formicans* may give the patterns of groups I, III, IV or V; *Plesiomonas shigelloides* gives the pattern of group VI; *Providencia*, group V, and *Pseudomonas aeruginosa*, group VI. Strains of *Aeromonas formicans* in particular may be wrongly identified as NCV's, on the basis of the Heiberg reactions.

Infrasubspecific tests

The positive VP reaction and production of haemolysin by El Tor vibrios were formerly considered the main criteria for differentiating these organisms from "classical" vibrios. However, haemolysin tests do not always give reproducible results and, moreover, many strains of El Tor vibrios from recent outbreaks have been either non-haemolytic or haemolytic only after serial subculture. Newer tests such as the first three in Table 4 are now being evaluated towards providing more reliable criteria, but strains have already been found which give an aberrant reaction in one or other of the

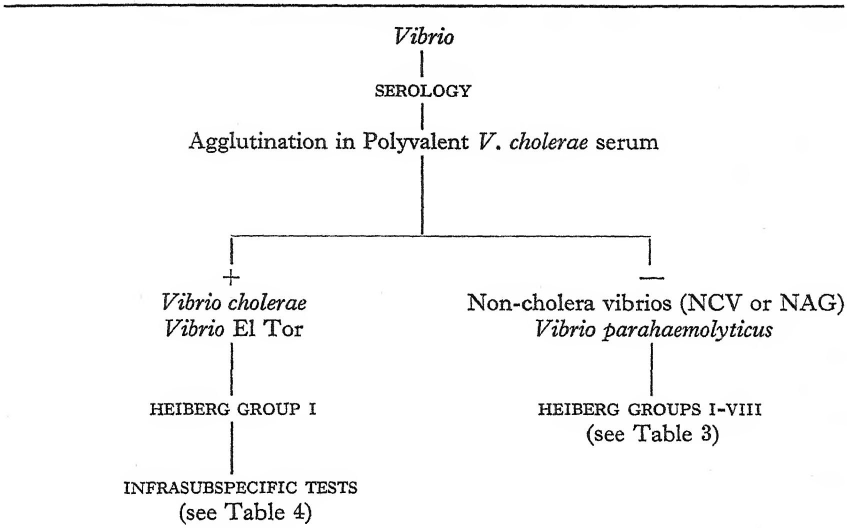

FIG 1. Vibrio subtyping

TABLE 3. Heiberg biogroups

	Mannose	Sucrose	Arabinose
Group I	+	+	—
Group II	—	+	—
Group III	+	+	+
Group IV	—	+	+
Group V	+	—	—
Group VI	—	—	—
Group VII	+	—	+
Group VIII	—	—	+

TABLE 4. Infrasubspecific characters of *Vibrio cholerae* and *Vibrio* El Tor

	Vibrio cholerae	*Vibrio* El Tor
Mukerjee group IV phage	sensitive	resistant
Chick cell haemagglutination	—	+
Polymyxin B 50 μg	sensitive	resistant
VP	—/+	+/—
Sheep cell haemolysis	—	+/—

tests. These more recently introduced tests are best done at a central rather than a local laboratory as adequate controls are necessary.

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Classification of Azotobacters

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Two characters serve to distinguish the azotobacters from other bacteria. These are the relatively large size of the individual cells, which may measure as much as 5μm in diameter as seen in wet preparations under the phase contrast microscope, and the ability of azotobacter cultures to fix atmospheric nitrogen when provided with a suitable energy source. Nitrogen-fixing ability is not, of course, restricted to the azotobacters, but few other groups of bacteria will fix amounts in excess of 10 mg of atmospheric nitrogen/g of carbohydrate consumed, as will the azotobacters. We would suggest, therefore, the following, rather tentative, working definition of the genus.

Azotobacter (Beijerinck, 1901)

Large rods or even cocci showing considerable variation in shape and size, sometimes almost yeastlike, often motile. Flagellation may be peritrichous or polar. Endospores not formed, but thick-walled microcysts produced by some species. The cells are usually described as Gram-negative, but some strains are Gram-variable and may even be Gram-positive. Obligate aerobes, capable of fixing atmospheric nitrogen when provided with a suitable carbohydrate or other energy source. Cultures grow best with free nitrogen, or simple forms of combined nitrogen such as ammonia. Catalase positive. Found in soil and water.

Although there are many species of azotobacter reported in the literature (Rubenchik, 1959), many of them are either inadequately described or are clearly minor variants of the six species which are currently accepted as valid members of the genus. The species which are fully described in the literature, and of which pure cultures are available for study are: *Azotobacter chroococcum* (Beijerinck, 1901); *Azotobacter agilis* (*agile*) (Beijerinck, 1901); *Azotobacter vinelandii* (Lipman, 1904); *Azotobacter beijerinckii*

TABLE 1. Characteristics of azotobacter species

Species	Average cell size	Microcyst formation	Flagellation	Pigments: Insoluble	Pigments: Soluble	Pigments: Fluorescent	Growth on starch	Habitat
Az. agilis	3–5μm diam.	—	Peritrichous	—	Green	Bluish white	N.R.	Water
Az. insignis	3–5μm diam.	—	Polar	Light brown	Green	—	N.R.	Water
Az. vinelandii	2·4μm × 0·5μm	+	Peritrichous	—	Green	Apple green	N.R.	Soil and water
Az. macrocytogenes	2μm diam.	—	Polar	—	Purple	N.R.	N.R.	Soil
Az chroococcum	2·4μm × 0·5μm	+	Peritrichous	Brown	—	—	+	Soil and water
Az. beijerinckii	2·8μm × 7·6μm	+	Non-motile	Light Brown -sepia	—	—	—	Soil and water

N.R. = Not relevant to classification.

(Lipman, 1904); *Azotobacter insignis* (*insigne*) (Derx, 1951); and *Azotobacter macrocytogenes* (Jensen, H. L., 1955). The differentiation of these species from one another is not normally difficult using relatively simple morphological and physiological tests. The characteristics of the species are summarized in Table 1 and, in our experience, it is rare for a newly isolated azotobacter to fail to fit into one of these species.

Methods

Glucose, nitrogen-free medium (*Norris*, 1959)

Azotobacters grow readily as surface pellicles on shallow layers of fluid medium of the following composition (per cent w/v): glucose, 1; K_2HPO_4, 0·1; $MgSO_4.7H_2O$, 0·02; $CaCO_3$, 0·1; NaCl, 0·02; $Na_2MoO_4.2H_2O$, 0·0005. The components are dissolved in distilled water and the medium sterilized by autoclaving at 121° for 10 min. Alternative carbon/energy sources may be used in place of glucose and it is convenient to prepare a basal medium to which sterile solutions of appropriate carbon/energy sources are added asceptically as required. The medium may be solidified by the addition of 2% agar.

Observation of cell morphology and motility

The appearance of cells from young (overnight) cultures grown in glucose nitrogen-free fluid medium at 30° is often of great assistance in the identification of azotobacters (Fig. 1). Cultures for microscopy are best prepared as wet preparations and observed by phase contrast. The inclusion of nigrosin or indian ink in the preparation is often useful, since it facilitates observation of the characteristic slime layers which surround cells of several species. Similar wet preparations are also used for observation of motility in young cells grown on fluid medium. The type of motility, smooth progression in one direction or zigzag circling movement, may be important in determining the species to which a particular culture belongs.

Pigment production

Two types of pigment are produced by azotobacters: non-water-soluble pigments which result in pigmented colonies, and water-soluble pigments which diffuse readily into agar media and are best seen in slope cultures.

Insoluble pigments

These may range in colour from pale cinnamon through various shades of sepia and yellow to dark brown. They develop slowly, tending to darken with age and reaching their maximum intensity after two weeks at 30°.

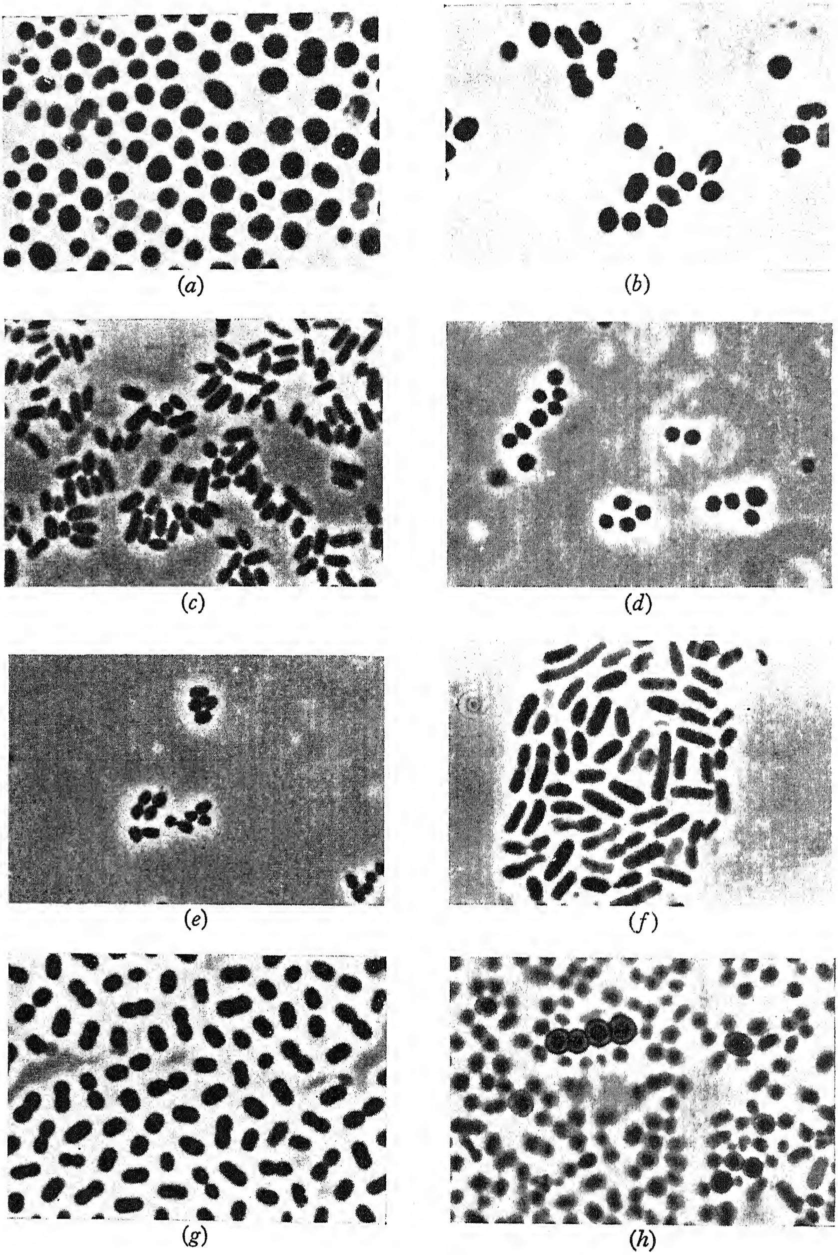

FIG. 1. Phase contrast photomicrographs of wet preparations of *Azotobacter* cells ($\times$ 1125). (*a*) *Az. agilis*; (*b*) *Az. insignis*; (*c*) *Az. vinelandii*; (*d*) *Az. macrocytogenes* (O form); (*e*) *Az. macrocytogenes* (M form); (*f*) *Az. chroococcum*; (*g*) *Az. beijerinckii*; (*h*) *Az. chroococcum* showing microcyst formation.

Colonies show good pigmentation when grown on glucose nitrogen-free agar.

Water-soluble pigments

The water-soluble pigments range in colour from greenish to purple and are most readily seen when cultures are grown for a few days on glucose, nitrogen-free agar at 30°. Variation in pigment-producing ability on different batches of medium is not infrequent and the reasons for this are not always clear. Iron in small quantities is detrimental to pigment formation and media for observation of water-soluble pigments should be prepared from glass distilled or deionized water. The ability of certain species to produce water-soluble pigments which fluoresce under ultraviolet light is an important diagnostic criterion. Cultures for this purpose are grown on glucose, nitrogen-free agar for 2 or 3 days at 30° and examined under ultraviolet light of wavelength 3600 Å. The colour of the ultraviolet fluorescence is significant. *Az. agilis* fluoresces a bright bluish-white colour; *Az. vinelandii* gives a distinctive apple green (Johnstone and Fishbein, 1956). New isolates should always be compared with cultures of known type.

Microcyst formation

In old cultures of some species of azotobacter the vegetative cells round off and develop a thick refractile wall to form characteristic microcysts. These are much more resistant than vegetative cells to the lethal action of ultraviolet light, sonic treatment and desiccation, although they do not have any significant thermal resistance. The ability to form microcysts is an important diagnostic character and is best determined by examining old cultures grown on glucose nitrogen-free agar, under phase contrast. When microcysts are transferred to fresh medium they germinate to reproduce as vegetative cells (Socolofsky and Wyss, 1961, 1962; Parker and Socolofsky, 1966).

Observation of type of flagellation

The observation of flagella type in azotobacters is complicated by the copious amounts of slime which some of the species produce. Cultures for observation are best grown for short periods, not exceeding 48 h, at 30° on agar slopes. The slopes are then gently covered with distilled water and allowed to stand at room temperature for about one hour to allow cells to swim away from the culture mass into suspension. Alternatively, cultures may be harvested in distilled water, stored in suspension overnight at 5° and washed three or more times in distilled water by slow centrifugation to remove capsular material. Following treatment in this way the flagella are

easily stained by the usual techniques, e.g. that described by Leifson (1960), or observed by electron microscopy (Baillie, Hodgkiss and Norris, 1962).

Characterization of the Species

The aquatic species

Only *Az. agilis* and *Az. insignis* appear to be solely aquatic species. These two organisms have been isolated from numerous fresh-water bodies and watercourses. *Az. agilis* is commonly found in water bodies containing polysaccharide waste material such as the effluent from strawboard mills. Both species grow as large oval cells some 3–5 μm in diameter. *Az. agilis* cells tend to be surrounded by more substantial slime layers than do those of *Az. insignis* and the colonies of *Az. agilis* growing on agar plates are bigger and more mucoid than those of *Az. insignis*.

Both produce water-soluble greenish pigments which diffuse into agar media and tend to darken as the culture ages sometimes becoming almost purple. Under ultraviolet light cultures of *Az. agilis* fluoresce with an intense bluish-white colour; cultures of *Az. insignis* are not fluorescent. Both species are motile and there is a marked difference in the type of motility seen in wet preparations of the two organisms. Cells of *Az. agilis* proceed with a smooth movement in one direction across a field; cells of *Az. insignis* on the other hand move with a characteristic zigzag circling movement. This difference in the nature of the movement of the cells reflects the fact that *Az. agilis* is peritrichously flagellate, whilst *Az. insignis* bears up to a dozen flagella in a polar tuft (Baillie, Hodgkiss and Norris, 1962). Neither species produces microcysts.

The soil-inhabiting species

Azotobacters are commonly found in cultivated soils and in soils where the pH is relatively high (e.g. above 6·5). They are conveniently isolated by enrichment techniques employing fluid nitrogen-free media in shallow layers and the enrichment cultures are purified by streaking on similar media solidified with agar. The commonest species isolated in this way are *Az. chroococcum* and *Az. beijerinckii*. *Az. vinelandii* appears to be a somewhat rarer organism and *Az. macrocytogenes* has been isolated from only two different sources.

Az. chroococcum and *Az. beijerinckii* are rather similar bacteria, the cells of *Az. chroococcum* measuring roughly 2·4μm $\times$ 5·0μm and those of *Az. beijerinckii* 2·8μm $\times$ 6·6μm. Both show considerable pleomorphism in old cultures. The formation of brown insoluble pigments is characteristic of these two species, colonies of *Az. chroococcum* frequently becoming very

dark brown in colour. *Az. beijerinckii* tends to produce less intense pigment ranging through various shades of light brown and cinnamon darkening to sepia. Completely unpigmented strains of both species are by no means uncommon. *Az. beijerinckii* normally produces more slime than *Az. chroococcum* and colonies of *Az. beijerinckii* develop, as the culture ages, a characteristic mucoid appearance. *Az. chroococcum* is vigorously motile in young cultures, but *Az. beijerinckii* is non-motile. *Az. chroococcum* grows readily on starch as energy source, but *Az. beijerinckii* will not grow on this substrate.

Az. vinelandii is readily distinguished from the other soil forms in that it produces a characteristic greenish water-soluble pigment resembling that of the aquatic species. It differs from the aquatic species in producing microcysts and in the behaviour of the cultures under ultraviolet light, where they fluoresce with a characteristic apple-green colour. The cells of *Az. vinelandii* are about the same size as those of *Az. chroococcum* and are vigorously motile in young cultures. Like *Az. chroococcum* cells, they are peritrichously flagellate.

Az. macrocytogenes is a much rarer organism and has been isolated from only two sites (Jensen, H. L., 1955; Norris and Baird, 1960). The young cells are spherical, roughly 2μm in diameter, and are often united in tetrads or cubes. They are feebly motile. Particularly large cells (6–8μm in diameter) are produced in cultures growing on ethanol as energy source. Capsule development is pronounced on solid media, capsules often showing a characteristic unilateral development. A pink, water-soluble pigment is produced which diffuses into agar media. One of the isolates fluoresces white under ultraviolet light; the other one does not fluoresce. *Az. macrocytogenes* is unusual in that acid is produced when cultures grow on glucose or sucrose. This may readily be detected in agar media containing pH indicators or by the incorporation of a little finely divided calcium carbonate into agar plates. This is dissolved in the region of developing colonies.

Both isolates of *Az. macrocytogenes* produce a stable variant form on culture in the laboratory. Colonies of this so called "M form" are smaller than those of the parent or "O form", and yellowish in colour. The cells of the variant form are lanceolate and are actively motile by one or more polar flagella.

Serological characterization of species

Although serological methods are not normally used in the identification and characterization of azotobacters, several workers have studied the antigenic composition of organisms in this genus. Studies of cell disintegrates show that all the species share at least one common intracellular antigen and that some species, such as *Az. agilis* and *Az. insignis* for example, share

a number of common antigens. Studies of saline extractable (capsular) antigens support the division into species outlined above. From studies of this kind *Az. agilis*, *Az. insignis* and *Az. vinelandii* emerge as clearly defined groups the members of which share common capsular antigens. The two isolates of *Az. macrocytogenes* are antigenically different from one another. There is considerable cross-reaction between different isolates of *Az. chroococcum* and *Az. beijerinckii* (Norris, 1960; Norris and Nagy, 1960).

Discussion

Although the identification of newly isolated azotobacters is a relatively simple matter and it is unusual for a new isolate to fail to fit neatly into one of the species described above, the overall taxonomy of the genus still presents considerable problems. The ability to produce microcysts is considered by some workers (Winogradsky, 1938; Tchan, 1953; Jensen, V., 1955) to be an important diagnostic criterion for the genus *Azotobacter*. Such considerations would lead to the restriction of the generic name *Azotobacter* to the species *Az. chroococcum*, *Az. beijerinckii* and *Az. vinelandii*. The non-microcyst-formers would then be accommodated in a second genus, which has been tentatively called *Azomonas* or *Azotococcus*. This genus would include *Az. agilis* and *Az. insignis*. Logically we should also need to include Jensen's *Az. macrocytogenes* in this second genus. Such a scheme certainly has the merit of including the two aquatic species, which are closely related serologically, in the same genus, but has the disadvantage that it would include both peritrichously and polarly flagellate species. An alternative approach is to transfer the two polarly flagellated species to a separate genus leaving the peritrichously flagellate and non-motile species in the original *Azotobacter*.

Of interest in this field are the observations of De Ley and Park (1966) on the DNA-base ratios of azotobacters. The base ratios of *Az. chrococcum*, *Az. beijerinckii* and *Az. vinelandii* are closely similar and it is suggested that these three species should constitute the genus *Azotobacter*. The ratios of *Az. beijerinckii* and *Az. chroococcum* are extremely close, suggesting that these may be variants of the same organism. Base ratio studies suggest that *Az. insignis* and *Az. macrocytogenes* should be regarded as a second genus for which the name *Azomonas* is suggested. *Az. agilis* DNA is different from the rest and this organism might represent a third genus, *Azotococcus*. Such a scheme has obvious merits, the microcyst-producing species would all be in the genus *Azotobacter*; the polarly flagellate species would appear together in the genus *Azomonas*; and the non-microcyst-forming but peritrichously flagellate *Azotococcus agilis* would be separated from both of these groups.

Future studies of the azotobacters may well lead to a splitting of the

group along lines similar to those mentioned above, but in our opinion our knowledge of these bacteria is insufficient to justify such a division at the present time. It seems advisable to us to retain the generic name *Azotobacter* for all of the six established species.

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Properties of *Acinetobacter* and Related Genera

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Aerobic Gram-negative bacteria which are non-pigmented, not polarly flagellate and do not ferment sugars present particular problems in identification, mainly because the genera in which they may be placed are so ill defined.

According to *Bergey's Manual* (Breed, Murray and Smith, 1957), such bacteria, if rod-shaped, should be placed either in the genera *Achromobacter* or *Alcaligenes* in the *Achromobacteriacae*, or in the genus *Agrobacterium* of the *Rhizobiaceae*. If coccoid, they would be placed in the *Neisseriaceae*. The unsatisfactory nature of criteria for separating *Achromobacter* from *Alcaligenes* has been stressed in the review by Ingram and Shewan (1960). The family *Rhizobiaceae* is separated from *Achromobacteriaceae* mainly on the ability to grow without organic nitrogen and the production of root nodules on plants (*Rhizobium*), and species of *Agrobacterium* may have properties very close to those of species of *Achromobacter*.

The genera mentioned include both peritrichous and non-motile species. In the low-temperature spoilage of protein foods, particularly poultry and fish, a group of non-motile strains with particular morphological properties is commonly isolated: these are cocci, coccoid rods or short rods, often found in pairs and staining either Gram-negative or sometimes Gram-variable. When isolated from foods, they have been described as either *Achromobacter*, *Alcaligenes* or as belonging to the *Achromobacter/Alcaligenes* group.

Similar morphological properties are described for bacteria from human sources such as *Bacterium anitratum*, *Moraxella* spp. and *Neisseria* spp. Although the pathogenic type species of *Moraxella* and *Neisseria* are well defined, strains resembling *Bact. anitratum* have appeared under a wide variety of names, such as *Herellea*, *Mima*, *Moraxella glucidolytica*, *Moraxella lwoffi*, *Achromobacter* and several others (see reviews by Rosebury, 1962; Thornley, 1967).

In order to clarify the taxonomic position of the non-motile coccoid rods

TABLE 1. Named strains from culture collections included in the survey

Strain No.	Designation and source
1	*Achromobacter anitratus* NCTC 8102 (B5W of Stuart, Formal and McGann, 1949)
2	*hartlebii* NCIB 8129 (ATCC 365; NRRL B-2392)
3	*lacticum* NCIB 8208 (NRRL B-551)
4	*lacticum* NCIB 8209 (NRRL B-552)
5	*venenosum* NCIB 9022
6	EB/F64/100 (Barnes)
7	Cb 11 (Holding)
8	7A14 (Holding)
9	A16 (Moore)
10	131 (Shewan)
11	138 (Shewan)
12	25A2 (Sulzbacher)
13	*Acinetobacter anitratum* NCIB 9019 (Strain 64 of Brisou, 1957)
14	*Aeromonas formicans* (Strain 18 of Eddy, 1960)
15	*Aeromonas liquefaciens* (*Pseudomonas hydrophila*) NCTC 7812 (Eddy, 1960)
16	*Agrobacterium tumefaciens* NCIB 8150 (ATCC 4720)
17	Lk10 (Holding)
18	Zh1 (Holding)
19	*Alcaligenes faecalis* NCTC 415
20	NCTC 8764
21	NCTC 655
22	NCTC 8769
23	sp.
24	*bookeri* NCIB 8155
25	*denitrificans* NCTC 8582 (Leifson and Hugh, 1954)
26	*viscosus* NCTC 3233
27	NCIB 8154 (ATCC 9036)
28	NCIB 8596
29	Cs8 (Holding)
30	Cs11 (Holding)
31	*Arthrobacter globiformis* NCIB 8602 (ATCC 4336)
32	*Bacterium anitratum* B5 (Ontario) Strain 705 (Schaub)
33	B7 (Ontario) Strain Eddy (Schaub)
34	B9 (Ontario) Strain Biol 2 (Schaub)
35	B10 (Ontario) (Strain 90 of Schaub and Hauber, 1948)
36	B11 (Ontario) (Strain 93 of Schaub and Hauber, 1948)
37	B16 (Ontario) NCIB 9293 (Strain B5W3 of Ferguson and Roberts, 1950)
38	B24 (Ontario) (Strain B5W72 of Ferguson and Roberts, 1950)
39	B25 (Ontario) NCIB 9301 (Strain B5W99 of Ferguson and Roberts, 1950)

40	*Cellulomonas biazotea*	NCIB 8077 (ATCC 486)
41	*Diplococcus mucosus*	169 (Klinge)
42		019 (Klinge)
43		So 1472/61 (Klinge)
44		So 1506/61 (Klinge)
45		E 2241/60 (Klinge)
46		E 8743/60 (Klinge)
47	*Escherichia coli*	NCTC 9001
48	*Herellea*	Z6 (Goldberg)
49		Z7 (Goldberg)
50		Z8 (Goldberg)
51		5937 (King)
52		5939 (King)
53		5942 (King)
54		5944 (King)
55		6009 (King)
56	*Mima*	Z1 (Goldberg)
57		Z2 (Goldberg)
58		Z3 (Goldberg)
59		Z4 (Goldberg)
60		5902 (King)
61		5936 (King)
62		5979 (King)
63	*Moraxella lwoffi*	NCTC 5866
64		NCTC 5867
65		NCTC 7976
66		950/56 (Klinge)
67		148/57 (Klinge)
68	*Neisseria catarrhalis*	NCTC 3622
69	*Pseudomonas aeruginosa*	NCTC 6750
70		NCTC 2000
71	*fluorescens*	NCTC 4755
72		NCIB 3756
73		28/5 (Rhodes) (NCTC 10,038; NCIB 9046; ATCC 13525)
74	*putrefaciens*	NCIB 8615
75	*saccharophila*	NCIB 8570 (ATCC 9114; NRRL B-1492)
	**Achromobacter liquefaciens*	ATCC 15716. (Tulecke *et al.*, 1965)

*This strain was studied after the computer survey was made.

Sources of strains:

Dr E. M. Barnes, Low Temperature Research Station, Cambridge.

Dr B. P. Eddy, Low Temperature Research Station, Cambridge.

Professor H. S. Goldberg, Department of Microbiology, University of Missouri, Columbia, Missouri, U.S.A. Strains of *Herellea* and *Mima* from human and animal sources.

Dr A. J. Holding, The Edinburgh School of Agriculture, Edinburgh 9. Strains of *Achromobacter*, *Agrobacterium* and *Alcaligenes* from soil.

Dr E. O. King, Communicable Disease Center, Public Health Service, U.S. Department of Health Education and Welfare, Atlanta, Georgia, U.S.A. Strains of *Herellea* and *Mima* from human sources.

Dr K. Klinge, Universität des Saarlandes, Medizinische Fakultät, Institut für Hygiene und Mikrobiologie, Homburg, Germany. Strains of *Moraxella lwoffi* received from Dr Klinge were isolated by Dr H. Flamm, Hygiene-Institut der Universität, Wien, Austria, and strains of *Diplococcus mucosus* were isolated from human sources by Dr F. Legler, Staatliche Bakteriologische Untersuchungs-Anstalt, Erlangen, Germany.

Dr H. B. Moore, Donald N. Sharp Memorial Community Hospital, San Diego, California, U.S.A.

Ontario Department of Health Laboratory, 360 Christie Street, Toronto 4, Ontario, Canada. Strains of *Bacterium anitratum* originally received from Dr I. Schaub, Johns Hopkins Hospital, Baltimore, Ma., and from Michigan Department of Health, Lansing, Michigan, U.S.A.

Dr M. E. Rhodes, Department of Botany, Coleg Prifysgol Cymru, Aberystwyth.

Dr J. M. Shewan, Torry Research Station, Aberdeen. Strains of *Achromobacter* from fish.

Dr W. L. Sulzbacher, Bureau of Animal Industry, Agriculture Research Administration. U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.

isolated from poultry, the author undertook a computer survey comparing a large number of these strains with representatives of all the genera just mentioned. The results showed that all the non-motile coccoid rods, whether originating from poultry or from human sources, formed groups which were distinct from those of the motile peritrichous rod-shaped forms. It is therefore suggested that generic separation would be useful; the genus *Acinetobacter* should be used provisionally for the non-motile coccoid rods, leaving the motile peritrichous rods in *Alcaligenes*, *Achromobacter* or *Agrobacterium*.

A brief report of this work is presented here; for further details, see Thornley (1967).

Results of the Computer Survey

All named strains included in the survey are listed in Table 1. These were compared with 120 strains isolated by the author, denoted by MJT/F4 or F5/, followed by the strain number.

Morphological and commonly used bacteriological and biochemical tests were included in the survey; full details of methods are listed by Thornley (1967). The author is indebted to Mr J. C. Gower and Mr G. S. J. Ross, who did the computing, using a similarity coefficient described by Gower (1968), with clusters formed by the method of single linkage. The term "phenon" is used as suggested by Sneath and Sokal (1962) to denote groups formed by the computer, and the prefix indicates the level of similarity at which strains are associated.

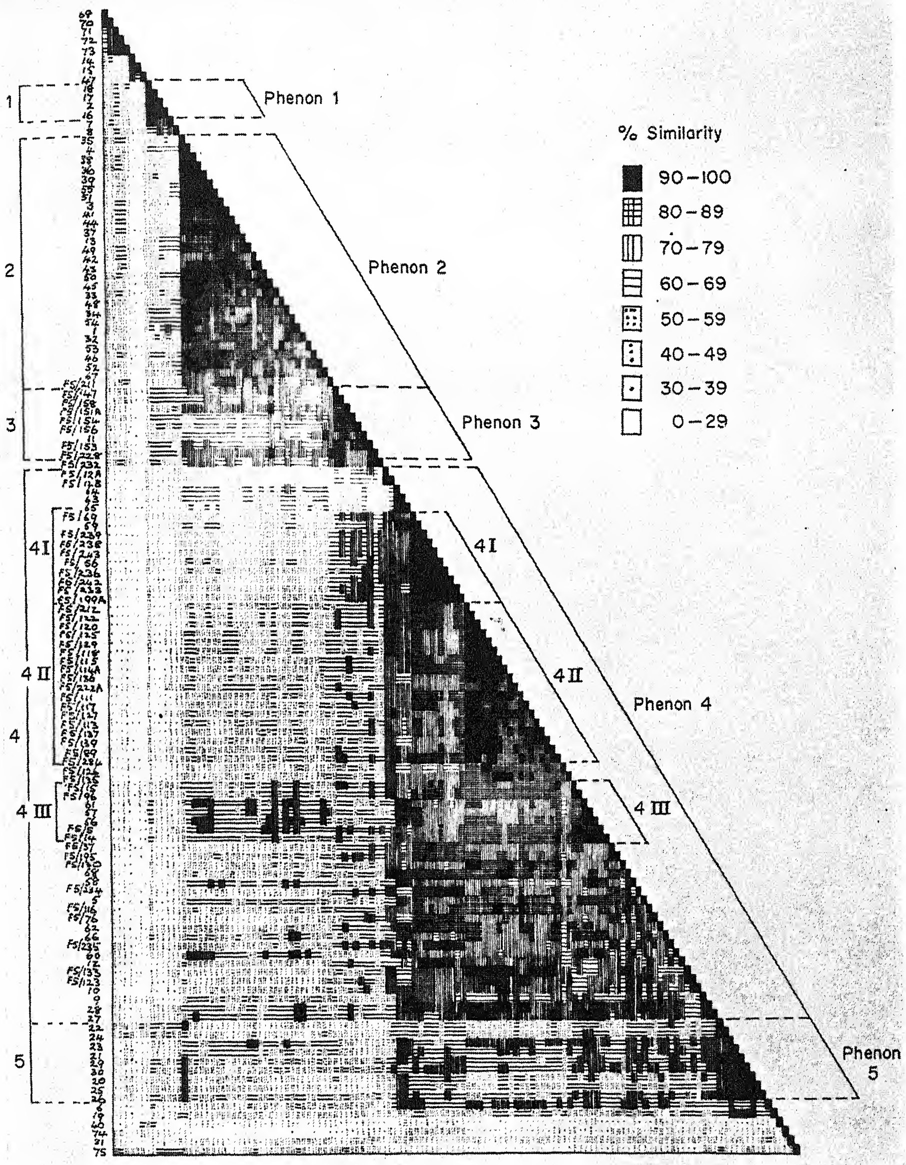


FIG. 1. Results of the first computation: similarity matrix. This shows similarity coefficients between every pair of strains, after arrangement of strains into groups ("phenons") by the computer. Strain numbers are listed at the left, and are arranged along the base in the same order.

The single numbers refer to named strains (see Table 1), while those prefixed by F5 are the author's isolates.

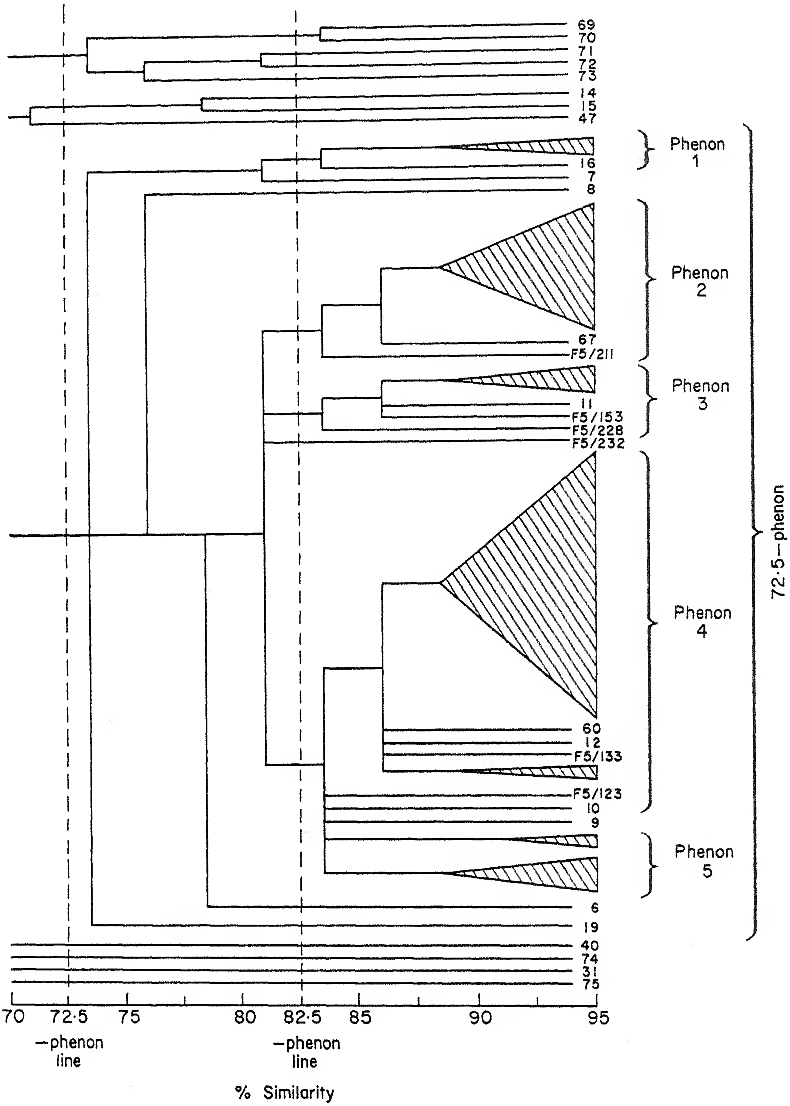


FIG. 2. Results of the first computation: simplified dendrogram. This shows the levels of similarity at which strains and phenons are linked. Most strains are grouped at 72·5% similarity into a very large phenon ("72·5-phenon"), further subdivided at approximately 82·5% similarity into phenons 1–5. Triangles represent all groups of strains associated at 87·5% similarity.

The data were handled in two computations, the first of which is illustrated by Fig. 1, representing the similarity matrix, and Fig. 2, which shows a simplified dendrogram of the results. In the second computation representative strains selected for each phenon of the first computation were tested against a different set of poultry isolates, obtained from a factory with different processing methods. These therefore represented an independent sample from the environment. Clusters were formed which could be identified with the phenons of the first computation by the representative strains, which were included in both.

The 72·5–phenon

From Fig. 2, the grouping of strains into phenons can be seen, and groups formed at two different similarity levels will be considered. First of all, at 72·5% similarity, all strains except twelve were associated; that is, they formed a 72·5–phenon. This contained only non-fermenting, non-pigmented strains, and other characters common to this very large group were also negative (Table 2).

The tests which differentiated the twelve strains outside the 72·5–phenon were, in general, those known to be useful for separation at generic level, including Hugh and Leifson's (1953) test for sugar utilization, pigmentation (King, Ward and Raney, 1954) and the arginine test (Thornley, 1960). The results for gluconate utilization were obtained with Paton's (1959) inorganic medium; where growth took place, reducing compounds were tested for with Benedict's reagent. This gave positive results only with strains of *Pseudomonas* and *Aeromonas*, suggesting that the reaction may be of some value for differentiating large groups of bacteria. However, the test would be more generally applicable if the gluconate were combined with a more complex medium so that all strains tested would grow.

Phenons 1–5 within the 72·5–phenon

The separation of the very large 72·5–phenon into smaller groups is shown in Fig. 2. In general the phenons marked 1–5 correspond to 82·5–phenons except for phenons 4 and 5, where the grouping has been based on mean similarity, as explained by Thornley (1967). Results for the second computation differed mainly in showing a closer relationship between phenons 3 and 4.

The position of each of the named strains can be seen by reference to Fig. 1 and Table 1, and a summarized version of this information, together with the representative strains (i.e. those with the highest mean similarity to the other strains in the same phenon) is quoted in Table 3. From the computer data the properties which had been most effective in separating

TABLE 2. Properties of strains

Properties of all strains in the survey

Gram-negative or variable rods, cocci or coccoid rods, able to grow aerobically on heart infusion or yeast extract agar at 25°.

Properties differentiating strains outside the 72·5-phenon

Properties	72·5-phenon (183 strains)	*Strains outside the 72·5-phenon*: 69 *Ps. aeruginosa*	70 *Ps. aeruginosa*	71 *Ps. fluorescens*	72 *Ps. fluorescens*	73 *Ps. fluorescens*	14 *Aeromonas*	15 *Aeromonas*	47 *E. coli*	40 *Cellulomonas*	74 *Ps. putrefaciens*	31 *Arthrobacter*	75 *Ps. saccharophila*
Gas from glucose	—							+	+				
Fermentation of:													
Glucose	—						+	+	+	+			
Galactose	—						+	+	+	+			
Xylose	—									+			
Arabinose	—						+	+	+	+			
Lactose	—						+		+	+			
Sucrose	—						+	+		+			
Fructose	—						+	+	+	+			
Glycerol	—						+	+	+	+			
Fluorescent pigment	—	+	+	+	+	+							
Colony colour:													
Yellow	—									+			+
Brown	—										+		
Reducing compounds from gluconate	—	+	+		+	+		+					
Arginine	— (98%)	+	+	+	+	+	+	+					
Digestion of serum	— (98%)	+	+			+	+	+			+		
Casein hydrolysis	— (98%)	+	+		+	+	+	+			+		
Starch hydrolysis	— (99%)						+	+		+			
Gelatine liquefaction	— (94%)	+	+	+	+	+	+	+		+	+	+	+

the phenons were found and are listed in Table 4. It is clear that a combination of properties is necessary to differentiate the phenons and that many results for the very large phenon 4 are variable. This reflects the existence within phenon 4 of smaller subgroups marked 4I–4III in Fig. 1; their properties are listed in Table 5.

TABLE 3. Strains included in phenons 1–5

Phenon	No. of strains	Representative strain	Original designation of strains included	
1	4	18 *Agrobacterium* Zhl	*Agrobacterium*	(3)
			Achr. hartlebii	(1)
2	28	39 *Bacterium anitratum* B25	*Bact. anitratum* *Achr. anitratum* *Acin. anitratum*	(10)
			Herellea	(8)
			Diplococcus mucosus	(6)
			Achr. lacticum	(2)
			Moraxella lwoffi	(1)
			MJT strain	(1)
3	23	MJT/F5/158	MJT strains	(22)
		MJT/F4/6/20	*Achromobacter* sp.	(1)
4	110	MJT/F5/111	MJT strains	(93)
			Mima	(7)
			Moraxella lwoffi	(4)
			Alc. viscosus	(2)
			Achr. venenosum	(1)
			Achromobacter sp.	(2)
			Neisseria catarrhalis	(1)
5	8	20 *Alc. faecalis* NCTC 8764	*Alc. faecalis*	(3)
			Alc. bookeri	(1)
			Alc. denitrificans	(1)
			Alc. viscosus	(1)
			Alcaligenes sp.	(3)

Evidence on Taxonomic Groups obtained from the Computer Survey

Although the point is perhaps rather obvious, it should be emphasized that the picture of relationships obtained from a computer survey cannot necessarily be translated directly into terms of taxonomy, because relevant groups of organisms may have been omitted from the survey. This is almost inevitable, especially with a wide range of organisms such as those studied here. Therefore, conclusions can be drawn only about groups which are adequately represented.

The most striking result found here has been the separation of motile and non-motile strains into separate phenons within the 72·5–phenon. All strains consisting of non-motile cocci, coccoid rods or short rods are placed in phenons 2, 3 or 4, while rod-shaped motile strains are included in phenons 1 or 5, or remain ungrouped in the 72·5–phenon. The way this separation applies to strains originally received as *Achromobacter* or *Alcaligenes* is illustrated in Figs 3 and 4.

TABLE 4. Properties differentiating phenons 1–5 within the 72·5–phenon (Properties without differential value have been omitted)

Properties		Percentage of strains giving the indicated results in phenon: 1	2	3	4	5
Number of strains		4	28	23	110	8
Morphology of cells		r	cr(75)sr(12) c (13)	cr(91)sr(9)	cr(88)sr(6) c (6)	r(88)sr(12)
Motility		+ (75)	—	—	—	+ (75)
Colonies		tr	op (86)	op (87)	op (70)	tr (75)
Oxidative acid production in Hugh & Leifson's medium with	glycerol	+ (75)	—	—	—	—
	sucrose	+	—	—	—	—
	fructose	+	—	—	—	—
	lactose	+	+ (96)	+ (96)	—	—
	arabinose	+	+	+ (96)	—	—
	xylose	+	+	+	—	—
	galactose	+	+	+ (96)	—	—
	glucose	+	+	+ (96)	—	—
H_2S in Kligler's medium		+	—	—	— (92)	—
Oxidase (Kovacs)		+	—	+ (96)	— (55)	+
NO_3 reduction		+ (75)	— (89)	+ (91)	— (55)	+ (88)
Growth on Simmons's citrate medium		+	+ (93)	— (78)	— (84)	+
Growth on Paton's gluconate medium		+	+ (68)	— (74)	— (51)	—
Growth at 0°		—	— (75)	+	+ (83)	— (88)
Growth at 37°		— (75)	+ (96)	— (87)	— (80)	+
Litmus milk— pH change		alk (50) ac (25)	ac (79)	uc(74)ac(26)	uc (88)	alk (75)
Penicillin sensitivity (2·5 i.u. tablets)		—	— (93)	+	+ (79)	—
DNA composition* (moles % guanine + cytosine)		58–64	37–41	44–45	37–46	57–67

Abbreviations: r=rod, sr=short rod, cr=coccoid rod, c=coccus, tr=translucent, op=opaque, alk=alkaline, ac=acid, uc=unchanged.

* Mandel and Thornley (to be published).

TABLE 5. List of all properties of strains in phenons 2, 3 and 4. These are all considered to be *Acinetobacter*, and phenon 2 represents *Acin. anitratus*

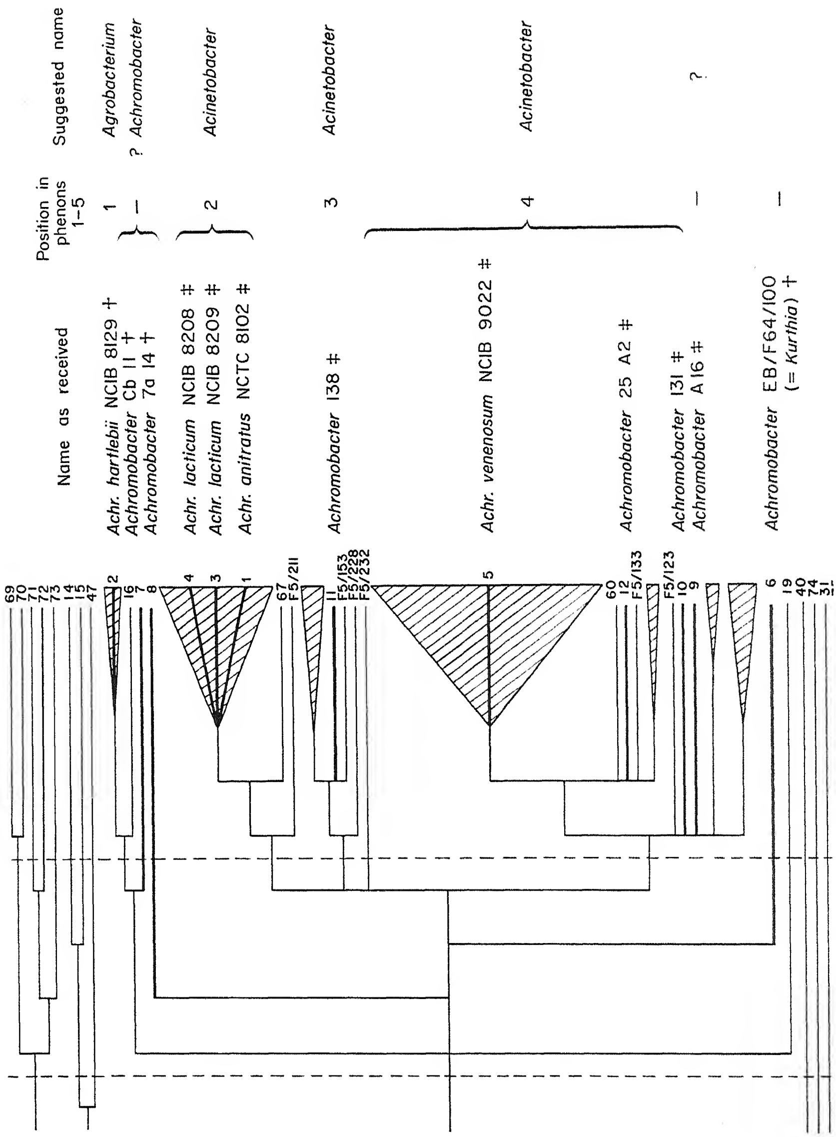
Properties common to all strains

Gram-negative or variable, non-spore-forming, non-motile coccoid rods, cocci or short rods, often in pairs; able to grow aerobically on nutrient media such as heart infusion agar or yeast extract agar; colonies whitish or cream, producing no fluorescent pigment; in Hugh and Leifson's (1953) test, no acid or gas is produced by fermentation, and sucrose, fructose and glycerol show no oxidative acid production (except for a few very slight changes); arginine test negative; nearly all strains failed to hydrolyse starch, casein or Löffler's serum and very few gave H_2S from Klingler's medium.

Properties not common to all strains

Property	Percentage of strains giving the indicated result in phenon:						
	2	3	4				
			4I	4II	4III	*Mor. lwoffi*	4, ungrouped
Number of strains	28	23	33	32	7	3	37
Oxidative acid production from 5 sugars*	+	+	—	—	—	—	—
Oxidase (Kovacs)	—	+ (96)	+	— (97)	—	—	+ (50)
Nitrate reduction	— (89)	+ (91)	+ (97)	—	— (71)	—	+ (41)
Growth on Simmons' citrate medium	+ (93)	— (78)	—	— (94)	— (57)	—	+ (35)
Growth on Paton's gluconate medium	+ (68)	— (74)	— (91)	+ (85)	+ (86)	—	— (54)
Growth at 0°	— (75)	+	+ (94)	+ (97)	+ (57)	—	+ (77)
Growth at 37°	+ (96)	— (87)	— (94)	—	— (57)	+	— (64)
Change of pH in litmus milk	ac (79)	uc (74) ac (26)	uc	uc	ac (71)	uc	uc (81) ac (14) alk (5)
Reduction of litmus milk	— (64)	+ (83)	+ (85)	— (66)	+	+	+ (49)
H_2S production from cysteine	— (64)	+ (57)	— (82)	+ (91)	+	—	+ (73)
Gelatine liquefaction	— (71)	—	—	— (97)	—	—	—
Greening of blood plates	+ (86)	—	—	—	— (71)	—	— (89)
Terramycin sensitivity (10 μg tablets)	+ (68)	+	+	+ (50)	+	+	+ (84)
Penicillin sensitivity (2·5 i.u. tablets)	— (93)	+	+ (97)	+ (87)	—	— (67)	+ (73)
Penicillin sensitivity (i.u./ml)	10–> 100	< 0·1	<0·1	1–10 (few 1–> 100)	10–> 100 (few 1–10)	nt	<0·1–20
DNA composition† (moles % guanine + cytosine)	37–41	44–45	44–46	40–43	37–40‡ 43–45§	nt	39–45

* Glucose, glactose, arabinose, xylose and lactose, tested in Hugh and Leifson's medium.
† Mandel and Thornley, to be published. ‡ Poultry isolates (MJT strains). § 3 strains of *Mima*. Abbreviations as in Table 4.


Name as received
Position in phenons 1–5
Suggested name
Achr. hartlebii NCIB 8129 +
Achromobacter Cb 11 +
Achromobacter 7a 14 +
1
—
Agrobacterium
? Achromobacter
Achr. lacticum NCIB 8208 ‡
Achr. lacticum NCIB 8209 ‡
Achr. anitratus NCTC 8102 ‡
2
Acinetobacter
Achromobacter 138 ‡
3
Acinetobacter
Achr. venenosum NCIB 9022 ‡
Achromobacter 25 A2 ‡
4
Acinetobacter
Achromobacter 131 ‡
Achromobacter A 16 ‡
—
?
Achromobacter EB/F64/100
(= Kurthia) +
—
69
70
71
72
73
14
15
47
2
16
7
8
4
3
1
67
F5/211
11
F5/153
F5/228
F5/232
5
60
12
F5/133
F5/123
10
9
6
19
40
74
31

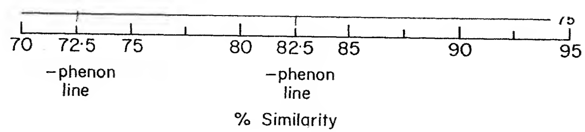


FIG. 3. Distribution of strains received as *Achromobacter*, shown on the dendrogram. † Motile; ‡ non-motile.

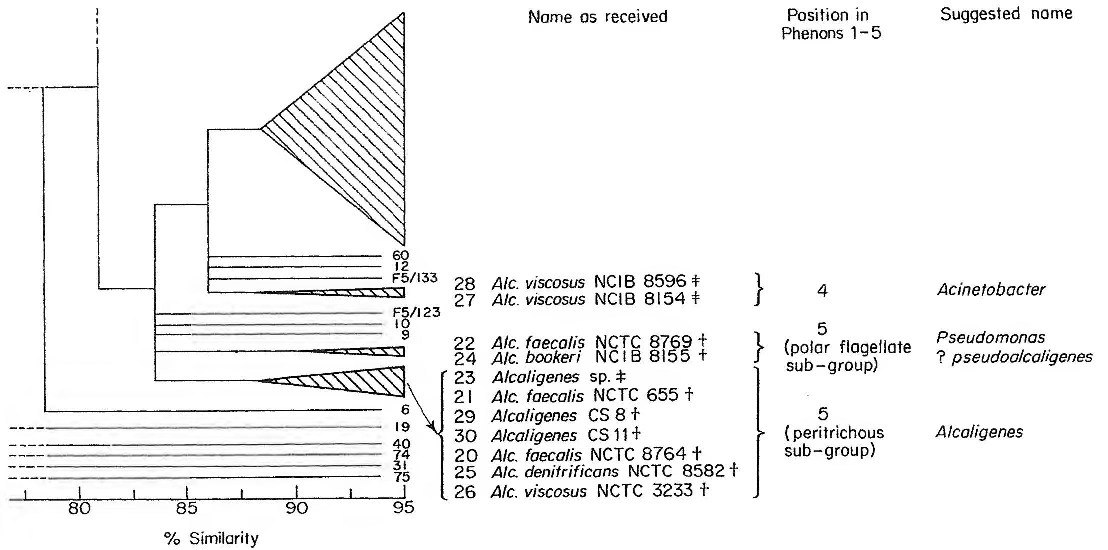


FIG. 4. Distribution of strains received as *Alcaligenes*, shown on part of the dendrogram.
† Motile; ‡ non-motile.

Data on the composition of the deoxyribonucleic acid of many strains in the survey was obtained by Mandel and Thornley (1967). The molar percentage of guanine plus cytosine varied between 37% and 46% for all the strains studied in phenons 2, 3 and 4, while it varied between 56 and 67% for strains in phenons 1 and 5, together with strains 7 and 8 (Table 4). This emphasized the difference between the motile peritrichous strains and the non-motile coccoid rods, already shown by their separation into different phenons. The two pieces of evidence taken together were considered to justify separation between the non-motile coccoid rods of phenons 2, 3 and 4 and the peritrichous rod-shaped organisms grouped in phenons 1 and 5 or remaining ungrouped within the 72·5–phenon.

On the computer results it might appear that phenons 2, 3 and 4 themselves should also be separated into different genera. This has not been done, because of relationships between phenons 2 and 3 and the subgroups of phenon 4, particularly close in the case of phenon 3 and phenon 41. The separation between these groups was mainly due to the oxidation of five sugars by phenon 3 (Table 5), and as these results might be due to associated enzyme systems it is felt that they are not satisfactory for generic separation. Moreover, the data on DNA composition do not support a wide separation between phenons 2, 3 and 4. In view of these facts, it is considered better at present to regard all the strains in phenons 2, 3 and 4 as belonging to the same genus, for which the name *Acinetobacter* seems the most appropriate.

Acinetobacter

Nomenclature

This has been discussed in detail by Thornley (1967). There are two main possibilities, *Moraxella* (Lwoff, 1939) and *Acinetobacter* (Brisou and Prévot, 1954). *Moraxella* is the earlier name, and strains of *M. lwoffi* were included in phenon 4. The use of this generic name for bacteria in phenons 2, 3 and 4 depends on the recognition of *M. lwoffi* as a valid member of the genus, which has been advocated by Piéchaud (1961) and Lwoff (1964) and opposed by Henriksen (1960), who prefers to place strains with the properties of *M. lwoffi* in *Acinetobacter*. Until further evidence is available the author suggests that *Acinetobacter* should be used.

Acinetobacter was proposed by Brisou and Prévot (1954) for non-motile strains previously called *Achromobacter* or *Alcaligenes*, and the genus has not been widely recognized, possibly because of doubts as to the validity of generic separation based mainly on non-motility. The present results provide much additional evidence for this generic separation.

The type species was *Acin. anitratus* (Brisou, 1957; Steel and Cowan, 1964) represented in phenon 2 in this work. Other species listed by Brisou

and Prévot (1954) and included here were *Acin. lacticum* (in phenon 2) and *Acin. lwoffi* (see Steel and Cowan, 1964) and *viscosus* (both in phenon 4).

Properties of the genus

Table 5 lists all the properties of strains in phenons 2, 3 and 4. The properties common to all strains, at the head of the table, can be regarded as a description of the genus *Acinetobacter*, as far as it has been studied here. It is disappointing that nearly all characters except morphology are negative, and it is hoped that future work will reveal positive biochemical features. The range in DNA composition found by Mandel and Thornley (to be published) for forty-six strains was 37–46 moles % guanine + cytosine.

Other features vary between phenons, but two of them may be useful for generic identification when positive. The pattern of sugar oxidation (glucose, galactose, arabinose, xylose and lactose oxidized; sucrose, fructose and glycerol not giving acid) found in phenons 2 and 3 (Table 4) has not been observed by the author for other Gram-negative bacteria, so may possibly be characteristic of this genus. Sensitivity to 2·5 i.u. of penicillin (Shewan, Hodgkiss and Liston, 1954) is found in phenon 3 and parts of phenon 4, and is unusual among Gram-negative bacteria apart from *Moraxella* and *Neisseria*.

Morphology

On Gram-staining, the *Acinetobacter* strains often appeared a darker red than is usual for other Gram-negative bacteria, possibly due to retention of some of the crystal violet. Some of the poultry strains, especially when freshly isolated, showed a mixture of Gram-positive and Gram-negative cells in smears. All *Acinetobacter* strains were non-motile.

By light microscopy, most strains were classified as coccoid rods, with smaller numbers regarded as cocci or short rods (Table 4, phenons 2, 3 and 4). Electron microscopy of negatively stained preparations revealed grooves between dividing cells (e.g. Fig. 5, G), and many strains previously classed as coccoid rods were found to be cocci. This applies to the strains shown in Figs 5, 6 and 8. Figure 7 illustrates a strain consisting mainly of coccoid rods, as judged by both light and electron microscopy. The cells were often grouped in pairs (Fig. 6), sometimes in short chains (Fig. 8), tetrads or small clumps (Fig. 5). Capsular material (Figs 5 and 6,C) was particularly well developed in Strain MJT/F4/8/3 (Fig. 6), and fimbriae (Figs 5, 6 and 7, F) were often present. Electron micrographs for strains named *Bacterium anitratum*, *Diplococcus mucosus* and *M. lwoffi* (Klinge, 1959; Ryter and Piéchaud, 1963) showed them to be coccoid rods or rods.

In a strain from poultry (MJT/F5/199A, in phenon 4I) which was studied in more detail, the direction of successive planes of division was

FIGS 5–8. Electron micrographs of intact cells, negatively stained with potassium phosphotungstate. The strains shown are classified by the author as *Acinetobacter*. The author is indebted to Mr R. W. Horne for the electron micrographs.

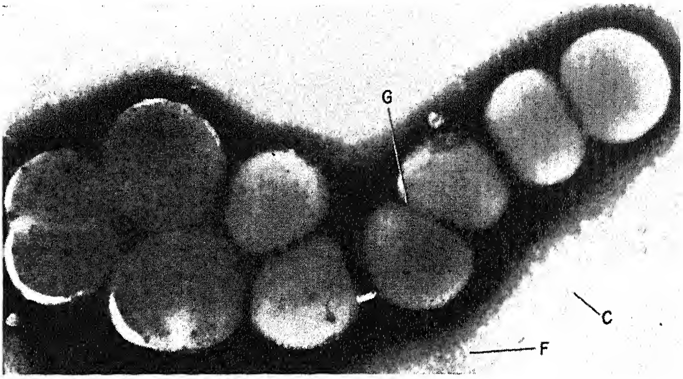


FIG. 5. Strain MJT/F5/158, the representative strain of phenon 3. Capsular material (C) and fimbriae (F) are present, and grooves (G) show the positions at which cell division is proceeding. × 16,000.

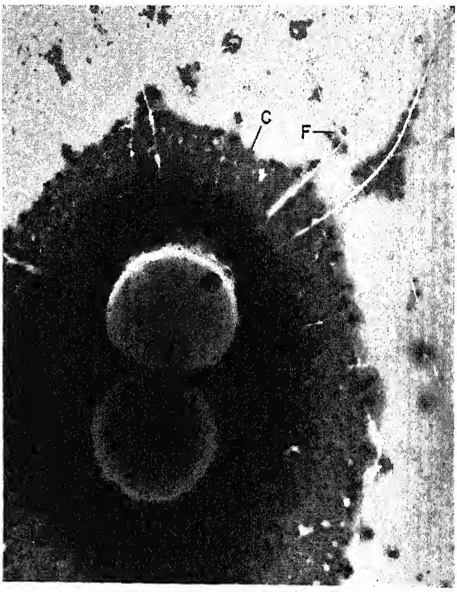


FIG. 6. Strain MJT/F4/8/3 in phenon 4, which consists of cocci in pairs with a very thick capsule (C) and long fimbriae (F). × 16,000.

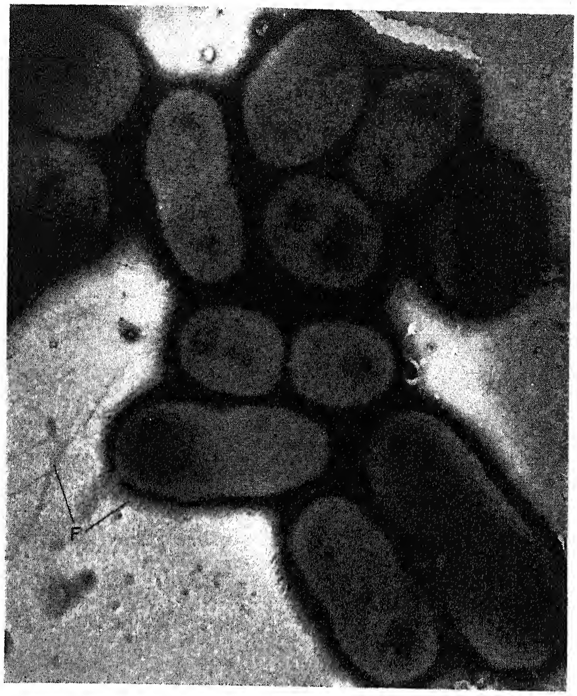


FIG. 7. Strain MJT/F5/5 in phenon 4III. The cells vary in shape from coccoid rods to short rods, and bear fimbriae (F). × 18,670.

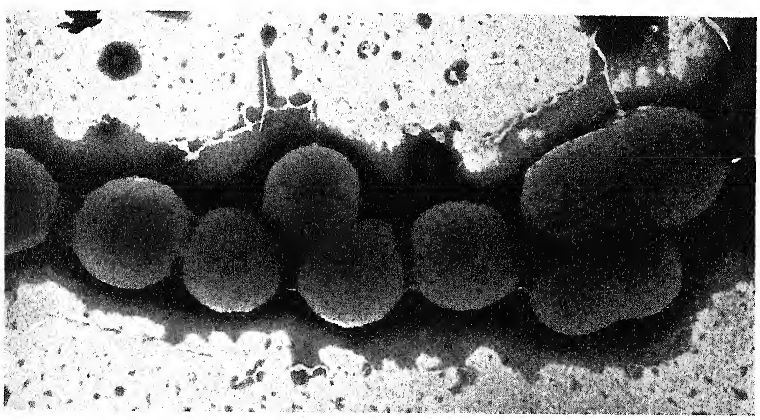


FIG. 8. *Alcaligenes viscosus* NCIB 8596, in phenon 4. The cocci are arranged in chains. × 16,000.

parallel in the logarithmic phase, giving rise to short chains, and perpendicular in the stationary phase, when tetrads were present (Thornley, 1967). This property is sometimes used for generic separation (e.g. between *Moraxella* and *Neisseria* (Piéchaud, 1961)), but clearly cannot be regarded as a generic character here.

The fine structure of various strains of *Acinetobacter* is now being studied by electron microscopy (Thornley and Glauert, 1968.)

Test differentiating phenons

The most useful tests are listed in Table 4, including two already mentioned for generic differentiation (p. 43). In addition, Kovacs (1956) oxidase test and nitrate reduction (of which the results seem to be correlated in this group of bacteria) give clear-cut separation between phenons 2 and 3, and between the small subgroups of phenon 4 (Table 5). Ability to grow on inorganic medium with citrate (Simmons, 1926) is common in phenon 2 (in agreement with the literature on *Bacterium anitratum*) and unusual in other phenons. Growth on inorganic medium with gluconate (Paton, 1959) is frequent in phenons 4II and 4III, while most strains in phenons 3 and 4I fail to grow in either Simmons's or Paton's media, possibly due to requirements for organic nitrogen or other growth factors. Obviously, more data on growth requirements are needed.

Species

Phenon 2 is thought to correspond to the type species, *Acin. anitratus*, since it includes many of the original strains described by the early workers on this species, then called *Bacterium anitratum* or B5W (see Table 1, strains 1, 32–39). This species has also previously been placed in *Achromobacter* and *Moraxella.* Other names of strains included in this phenon can be regarded as synonyms. This applies to *Herellea*, *Achr. lacticum* and *Moraxella lwoffi var. glucidolytica* (or *M. glucidolytica*) and also to *Diplococcus mucosus*, when this name is applied to strains with the properties of phenon 2. (It has also been used for strains with different properties, now thought to be *Neisseria.*) Many earlier studies, reviewed by Thornley (1967), have shown resemblances between strains with the above names, and these are confirmed by the present work.

Phenons 3 and 4 are not suggested for specific rank, since it seems quite likely that more detailed study could lead to rearrangement , e.g. phenons 3 and 4I might be merged through the knowledge of additional characters. However, the same phenons (with the exception of the very small phenon 4III) emerged in two computations using poultry isolates from different sources, so it seems that they must represent naturally occurring groups.

Alcaligenes

The type species of this genus is the motile peritrichous *Alc. faecalis* and two named strains of this were grouped with four strains of similar properties within phenon 5, the other small subgroup of phenon 5 consisting of two polar flagellate strains, originally called *Alcaligenes*. Two strains of the non-motile *Alc. viscosus* were placed in phenon 4 and have therefore been dealt with as *Acinetobacter* (Fig. 4).

It has been suggested by Brisou and Prévot (1954), Hendrie, Hodgkiss and Shewan (1964) and others, that after separation of non-motile strains in *Acinetobacter*, the remaining motile strains of both *Alcaligenes* and *Achromobacter* should be grouped together as *Achromobacter*. The present author feels that the existence of a group of strains resembling *Alc. faecalis* makes the genus *Alcaligenes* far more satisfactory than *Achromobacter* in which the type species cannot be recognized. It is suggested therefore that *Alcaligenes* should be retained for strains resembling *Alc. faecalis*.

The properties of phenon 5 are listed in Table 4, but for more comprehensive results on *Alc. faecalis* see Board (1965).

The two polarly flagellate strains (Table 1, 22 and 24; see Hendrie *et al.*, 1964) differed from the peritrichous group in a few properties, including lack of alkalinity in litmus milk and development of a very slow arginine reaction; they might be *Pseudomonas alcaligenes* (Hugh and Ikari, 1964) or *Ps. pseudoalcaligenes* (Stanier, Palleroni and Doudoroff, 1966). Some confusion has arisen in the past from the inclusion of both polar and peritrichous strains in *Alcaligenes*, but it seems likely that the two groups could in future be more clearly resolved by using additional tests such as those of Stanier *et al.* (1966) and Board (1965).

Agrobacterium

Only three named strains of *Agrobacterium* were included in the survey and these grouped together with Strain 2, *Achr. hartlebii* NCIB 8129 in phenon 1, suggesting that the latter should be considered for possible inclusion in *Agrobacterium*.

The properties of phenon 1 included oxidation of both sucrose and fructose in Hugh and Leifson's medium, and production of H_2S in Kligler's medium, in which this phenon differed from the rest of the 72·5–phenon (Table 4). However, it is not known whether these properties are widespread in the genus *Agrobacterium*.

Achromobacter

Most of the strains originally designated *Achromobacter* and included in this survey were non-motile, fell into phenons 2, 3 and 4 (Fig. 3) and are now considered to be *Acinetobacter*.

Since the type species of *Achromobacter* (*Achr. liquefaciens*) is described as motile and peritrichous (Breed *et al.*, 1957), it seems reasonable that strains having these properties should be considered for inclusion in a revised version of the genus. However, only four such strains were studied, and these were scattered in distribution (Fig. 3); Strain 2 (already mentioned) fell into phenon 1, Strain 7 close to phenon 1, and Strain 8 in the 72·5–phenon, but not close to any group. Strains 7 and 8, isolated from soil by Dr A. J. Holding, may therefore be suitable for inclusion in a revised *Achromobacter*, but the borderline between this and *Agrobacterium* obviously needs clarification.

Strain 6 was motile, peritrichous and Gram-variable, and later proved to belong to the Gram-positive genus *Kurthia* (Dr T. Gibson, pers. comm.).

Tulecke, Orenski, Taggart and Colavito (1965) proposed a neotype strain of *Achromobacter liquefaciens*, ATCC 15716. This was studied after the computer survey was finished, and found to differ in a large number of characters from all strains in the survey. It was Gram-variable, fermented several sugars and was thought likely to be closer to a Gram-positive genus (Thornley, 1967). Electron microscopy of sections of the bacteria showed that the cell-wall structure was typical of a Gram-positive bacterium (Thornley and Glauert, unpublished). Therefore, although this organism fits Eisenberg's (1891) original description of *Bacillus liquefaciens*, it does not seem suitable to represent the type species of a Gram-negative genus.

Many other motile peritrichous strains of *Achromobacter* exist in culture collections (see Surdy and Hartsell, 1963; Hendrie, Hodgkiss and Shewan, 1964) and need further study to show whether the genus *Achromobacter* should be considered valid.

The author is grateful to J. C. Gower and G. S. J. Ross who did the computing and to R. W. Horne for the electron micrographs.

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The Identification and Classification of *Rhizobium*

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The need to identify strains of rhizobia that nodulate legumes, especially those important to agriculture, has led to the grouping of strains into several species. The methods available for classifying nodule bacteria in this way are outlined below. However, there are reasons for thinking that this practically useful grouping is unsound taxonomically and does not indicate the phylogenetic relationships of *Rhizobium*.

The genus *Rhizobium*

Rhizobium is a genus of aerobic heterotrophic non spore-forming soil bacteria able to invade the roots of leguminous plants and form nodules in which atmospheric nitrogen may be fixed. This ability to form root nodules is the most important characteristic distinguishing *Rhizobium* from other bacterial genera.

Rhizobium cells are small to medium sized (0·5–0·9 × 1·2–3·0μm) Gram-negative rods, often motile when young by polar, subpolar or peritrichous flagella. Cells contain characteristic granules of polymerised β-hydroxy-butyrate which stain with Sudan Black (Burdon, 1946; Schlegel, 1962), and appear as highly refractile bodies by phase contrast illumination. Most strains produce abundant extracellular polysaccharide slime that ranges from a watery consistency to a highly tenacious gum, the composition of which varies with the strain (Humphrey and Vincent, 1959).

The bacteria usually undergo morphological changes within the cells of

the nodule to form "bacteroids". These are branched or enlarged cells unable to grow on media that support growth of the unmodified cells (Fred, Baldwin and McCoy, 1932; Almon, 1933). Bacteroids are enclosed in membranes of host plant origin, sometimes singly and sometimes in groups. The arrangement of bacteroids within membranes may be of taxonomic value (Dart and Mercer, 1966). In nodules that fix nitrogen the bacteroid zone is pink because of leghaemoglobin that accumulates in the plant cells.

Cultural characteristics

Rhizobia can use a wide range of carbohydrates. Nitrogen is not fixed in culture. Inorganic combined nitrogen (ammonium or nitrate) usually suffices for growth. Some strains require growth factors (Wilson, 1940) and amino acids for optimal growth (Jordan, 1956; Bereśniewicz, 1959; Graham, 1963*a*; Strijdom and Allen, 1966) or can use other forms of organic nitrogen (Jensen and Schrøder, 1965). Nitrates but usually not nitrites are reduced (Jordan and San Clemente, 1955). Rhizobia are weakly proteolytic. Major inorganic elements are usually added even to complex media, but except for magnesium (Norris, 1958; Vincent, 1962), calcium (Vincent and Colburn, 1961) and cobalt (Lowe and Evans, 1962), little has yet been done to define these requirements. Synthetic media suitable for the growth of some strains have been developed by Ferry *et al.* (1959), Schwinghamer (1960), Bergersen (1961) and others, and calcium gluconate (5% w/v) and specific carbohydrate sources are useful media supplements for slow growing strains. The optimal temperature range is *c.* 25–30° (Bowen and Kennedy, 1959). A temperature of 70° for 10 min is lethal, as is drying under certain conditions, and the bacteria are usually intolerant of acid reaction (optimum pH 6–7). Strains differ in their resistance to antimicrobial agents (Davis, 1962; Golebiowska and Kaszubiak, 1965).

Cultural tests help to distinguish *Rhizobium* from other genera but are not diagnostic. Useful media for these tests are as follows.

Yeast extract mannitol agar (*YMA*) (based on Fred, Baldwin and McCoy, 1932). Mannitol, 10g; K_2HPO_4, 0·5g; $MgSO_4 7H_2O$, 0·2g; NaCl, 0·1g; $CaCO_3$, 4·0g; yeast water, 100 ml or yeast extract (e.g. Difco, Oxoid), 0·4g; agar 15g; distilled water to 1 l. The pH is adjusted to 6·8–7·0 and the medium autoclaved at 121° for 15 min. Calcium carbonate is usually omitted from medium used for making plates and from liquid medium when measuring growth turbidimetrically. The yeast water is prepared as follows: 100g of bakers' compressed yeast is mixed with 1 l. of cold water, allowed to stand at room temperature for 1–2 h and steamed for 40–60 min. The clear supernatant fluid after settling or centrifuging is autoclaved at 121° for 15 min and used as yeast water.

Rhizobia grow well on this medium, which is usually used for their routine cultivation, but even the faster growing strains take 3–5 days to produce abundant growth. Growth is generally colourless to white with more or less gum.

YMA with congo red. A 1/400 aqueous solution of congo red is sterilized separately and 10 ml added aseptically to 1 l. of the sterile melted YMA before tubing or use (Hahn, 1966).

Most strains of rhizobia fail to absorb congo red from this medium within a week of incubation though some will absorb a little. Marked absorption giving vivid red growth is characteristic of related genera (see *Agrobacterium*).

Glucose peptone agar. Glucose, 10g; peptone, 20g; NaCl, 5g; agar, 15g; pH, *c.* 7·2. Autoclave at 121° for 15 min. Most rhizobia grow poorly and show no growth in 24 h on this medium or other peptone media in routine laboratory use. There are exceptions to this, e.g. many strains of *Rhizobium meliloti.*

Litmus milk. Rhizobia produce slow changes in litmus milk, mainly towards slight alkalinity though a few strains of *R meliloti* give a slightly acid reaction. Many strains slowly digest the top layer of milk to form a "serum" zone.

Differentiation of Rhizobium *and* Agrobacterium

It is not always easy to distinguish *Rhizobium* from *Agrobacterium,* to which it is probably closely related. *Agrobacterium radiobacter* is a common soil bacterium and produces colonies on YMA indistinguishable from those of the faster growing species of *Rhizobium.* The other media mentioned above are useful for distinguishing between strains of these two genera. Species of *Agrobacterium* (e.g. *A. radiobacter A. rhizogenes* and *A. tumefaciens*) form a well defined group and typically give similar reactions. Transformation has been reported between *Rhizobium* spp. and *Agrobacterium tumefaciens* (Klein and Klein, 1953; Kern, 1965). All are fast growers on YMA, absorb congo red and grow on YMA containing 2% of NaCl. Some strains of *Rhizobium* will absorb a little congo red and others will grow with 2% of NaCl, but it is rare for a strain to do both.

Graham and Parker (1964) who surveyed many strains and used many cultural tests, pointed out that *Agrobacterium* spp. typically grow on glucose peptone agar, utilize citrate, produce hydrogen sulphide in bismuth sulphite medium and form precipitates in calcium glycerophosphate agar. By contrast, rhizobia typically make little or slow growth on glucose peptone agar, do not utilize citrate, form little or no H_2S from bismuth sulphite and give no precipitate in glycerophosphate agar. Strains of *R. meliloti* often behave like *Agrobacterium* strains in the tests made with these media.

The species of *Rhizobium*

Species of *Rhizobium* differ in their host range and, whereas some are restricted to one species of host plant, some are not. Isolates like those from *Trifolium pratense* (red clover) nodules which induce nodule formation on most other clover species but not, for example, on *Lupinus* spp. (lupins) or *Phaseolus* spp. (beans), are said to display cross-inoculation group specificity. Six major cross-inoculation groups of host plants given in Bergey's Manual (Breed, Murray and Smith, 1957) are widely accepted and the bacteria associated with each group are regarded as comprising a species. These, together with typical hosts in the corresponding groups of plants, are listed in Table 1.

The cross-inoculation groups of hosts given in Bergey's Manual contain very different numbers of plant species. The *Trifolium* group consists of one genus of host plants only, whereas the soybean (*Glycine max*) and cowpea (*Vigna sinensis*) groups together contain most genera of the three major subfamilies of the Leguminosae. Suitable test plants can be recommended for determining *Rhizobium* species for all groups except the *R. japonicum* and cowpea rhizobia (*Rhizobium* spp. in the Bergey classification), which present a special problem because of the very many host plants that they infect. Strains that nodulate cowpea often nodulate soybean. *R. japonicum* and cowpea rhizobia are sometimes referred to as the "soybean–cowpea miscellany". The short list of test plants for these groups (Table 1) includes only some host species that have proved useful.

Cross-infection (confirmed by reisolation and identification of strains) has been recorded between the lupin, phaseolus, soybean, and cowpea groups. Between the pea and clover groups cross-infection is sporadic and uncommon, and strains of *R. meliloti* infect the other host groups very rarely indeed. Usually strains are ineffective in fixing nitrogen on their unaccustomed host.

Tests for nodule formation

The principle of testing is to grow sterile host plants in sterile nitrogen free medium with and without the *Rhizobium* strain under test, and to observe whether nodules are formed in the presence of the bacteria. The test can also show whether or not nitrogen is fixed in the nodules.

Small seeded plants (e.g. *Trifolium pratense*) can be grown wholly enclosed within glass test tubes fitted with cotton wool plugs to protect them from contamination. Clean uniform seed is rinsed with 95% ethanol and immersed for 3 min in 0·2% (w/v) $HgCl_2$ solution. The seed is then washed thoroughly with at least 10 changes of sterile water and planted directly on agar slopes in tubes, or set to germinate on moist filter paper or

TABLE 1. Species allocation of *Rhizobium* based on nodulation

Test Host	*R. leguminosarum*	*R. phaseoli*	*R. trifolii*†	*R. lupini*	*R. japonicum*	Cowpea rhizobia	*R. meliloti*
**Vicia sativa* L., *V. hirsuta* L., S. F. Gray or *Pisum sativum* L.	+	±	±	—	—	—	—
Phaseolus vulgaris L.	$\underline{\underline{\pm}}$	+	$\underline{\underline{\pm}}$	$\underline{\underline{\pm}}$	±	—	—
**Trifolium repens* L.	±	$\underline{\underline{\pm}}$	+	—	—	—	—
**Ornithopus sativus* Brot.	—	—	—	+	—	±	—
**Phaseolus atropurpureus* Desf. (‡)	—	±	—	+	±	+	—
Glycine max Merr.	—	—	—	$\underline{\underline{\pm}}$	+	±	—
**Lotus uliginosus* Schkuhr.	—	—	—	±	—	+	—
Vigna sinensis Endl. (§)	—	—	—	—	—	+	—
**Medicago sativa* L. (¶)	—	—	—	—	—	$\underline{\underline{\pm}}$	+

+ = Always nodulates; — = not shown to nodulate; ± = sometimes nodulates; $\underline{\underline{\pm}}$ = very rarely nodulates; * = small seeded species that will nodulate in test tube culture.

† Note, however, low compatibility amongst isolates from Mediterranean species of *Trifolium*, *Trifolium ambiguum*, and African clovers in respect of their non-homologous host.

‡ *Phaseolus atropurpureus* is widely susceptible to a miscellaneous collection of rhizobia, some of which are otherwise very host specific (e.g. rhizobia from *Lotononis bainesii* and from *Leucaena* spp.).

§ *Vigna sinensis* (cowpea) is nodulated by isolates from many genera and species of legumes and occasionally by isolates of other *Rhizobium* spp.

¶ Lucerne (*Medicago sativa*) appears to be always invasible by *R. meliloti*. Other species of *Medicago* are likely to be more strain specific.

water agar (1·2%) in petri dishes at an appropriate temperature. Some legume seeds need temperature pretreatment before they will germinate (Crocker and Barton, 1953; Mayer and Poljakoff-Mayher, 1963). Hard coated seeds can be surface-sterilized by immersion in concentrated sulphuric acid for 20–30 min (seed must be dried over a desiccant), then washed quickly and thoroughly with up to 10 changes of sterile water after having drained off excess acid. This treatment also softens the seed coat and facilitates imbibition of water. Sterile seeds or germinated seedlings are transferred to slopes of sterile seedling agar in suitable sized test tubes (150 × 20 mm for the smallest seeded host plants) fitted with cotton wool plugs. A suitable seedling agar (Jensen, 1942) has the following composition: $CaHPO_4$, 1g; K_2HPO_4, 0·2g; $MgSO_4{\cdot}7H_2O$, 0·2g; NaCl, 0·2g; $FeCl_3$ 0·1g; agar, 8–15g; distilled water, 1 l. The pH is adjusted to 6·5 and the medium sterilized at 121° for 15 min.

The slopes can be inoculated with bacteria either immediately or within 7 days. It is convenient to arrange the tubes in racks so that the roots are partially shaded from direct light, and to put them in a glasshouse with precautions against overheating in the sun (Hely, 1959), or in an illuminated growth cabinet. The optimal temperature for nodulation depends on the legume used (Gukova, 1962; Pate, 1962; Gibson, 1963; Possingham, Moye and Anderson, 1964; Dart and Mercer, 1965). Slopes require the periodic addition of sterile water.

Nodules develop when the plants are 2–4 weeks old and the first true leaves are opening. Roots of some legumes, e.g. of species of *Medicago*, free from rhizobia, sometimes form outgrowths that superficially resemble nodules. Such pseudo-nodules (and tumours and galls produced by other bacteria, nematodes and insects) can be distinguished from true root nodules by their structure. Nodules have a peripheral vascular system and a definite endodermis surrounding the infected region containing the bacteria and bacteroids; the bacteroids stain readily with toluidine blue O (O'Brien, Feder and McCully, 1964).

The seeds of larger seeded species such as *Phaseolus vulgaris* L., *Lupinus luteus* L. and *Glycine max* Endl. can be surface sterilized with $HgCl_2$, as described above, and sown in sterilized sand or vermiculite in sterile pots. The risk of contamination with other soil organisms including rhizobia may be minimized by covering the sand surface with a 1–2 cm layer of waxed sand (Van Schreven, 1959). Plants are watered with sterile water or sterile nitrogen-free nutrient solution (e.g. the mineral salts of Jensen's (1942) seedling agar at half- or quarter-strength) through a plugged or capped glass tube passing through the waxed sand layer. Open pots should be used only when widely spaced on a clean glasshouse bench, and with enough controls to give a reliable check on contamination. Alternatively, the risk of

contamination can be lessened by using one of the several forms of "bottle-jar" units derived from the assembly of Leonard (1943). Date and Vincent (1962) and Norris (1964) have described suitable methods for making these tests with large seeded host plants.

Cultural characteristics of species of Rhizobium

The media referred to above can help to distinguish between rhizobia and other soil bacteria but little success has attended efforts to use cultural methods to identify *Rhizobium* species.

The rhizobia can be separated into two groups, depending on their rates of growth. *R. leguminosarum*, *R. phaseoli*, *R. trifolii* and *R. meliloti* in general produce abundant growth in 3–5 days at *c.* 25°, whereas *R. lupini* and *R. japonicum* grow much more slowly. There are notable exceptions to this generalization; some isolates from *Leucaena* nodules (cowpea group) grow as fast as *R. meliloti* and there are non-gummy varieties of *R. trifolii* that produce small colonies in culture. Most strains of these species fail to absorb congo red from medium containing it and do not grow on YMA containing 2% (w/v) of NaCl. Table 2 summarizes the morphological, cultural and symbiotic characteristics of the *Rhizobium* species, and also lists characteristics that exclude *Rhizobium*. Non-infective strains of *Rhizobium* that have lost the power to invade leguminous roots are very difficult to distinguish from other soil bacteria, especially *Agrobacterium*.

Strain Differences within Species

Symbiotic differences

Strains of nodule bacteria can be differentiated by their ability to form nodules on particular host species, by the nitrogen fixing effectiveness of the nodules they form and by other characteristics such as nodule number. These differences, particularly symbiotic effectiveness, can be used to subdivide the species of *Rhizobium*. For example, strains of *R. trifolii* have been provisionally separated into four sub-groups (Vincent, 1954; Hely, 1957; Norris and t'Mannetje, 1964; Nutman, 1965), and strains of *R. meliloti* into eight or nine subgroups (Brockwell and Hely, 1961, 1966). For references to the very extensive literature on symbiotic specificity in other species of *Rhizobium* see Vincent (1954), Nutman (1956), Allen and Allen (1958), Lange and Parker (1960, 1961), Gavigan and Curran (1962), Jensen (1964), Abdel Ghaffar and Jensen (1966), Brockwell, Hely and Neal-Smith (1966) and Brockwell and Neal-Smith (1966). Whilst some patterns can be seen so far as ability to form nodules is concerned, those related to nitrogen fixation are difficult to establish.

TABLE 2. Characteristics of *Rhizobium*

Test	Conformable characteristics	Contra-indications
A. *Morphology and staining*	Short-medium rods (1–3μm commonly) motile when young, often with prominent granules of polymer of β-hydroxybutyrate. Endospores absent. Gram-negative	Cocci, very large rods, long chains; endospores; Gram-positive
B. *Cultural*		
1. Growth on yeast mannitol agar 25°–28°	Little if any detectable growth in 24 h	Marked growth 1–2 days
	Moderate to abundant growth in 3–5 days, colourless or white, generally moderate to abundant gum **Rhizobium trifolii*, *R. leguminosarum*, *R. phaseoli*, *R. meliloti*; some "cowpea rhizobia"	Colour other than white (except pink for *Lotononis* strains)
	OR	
	Little growth after 5 days; slight to moderate growth in 10 days, colourless, white or rarely pink, slight gum production *R. lupini*, *R. japonicum*, *Lotus* rhizobia and most cowpea rhizobia; *Rhizobium* of *Lotononis bainesii*, characteristically pink	
2. Growth on congo red yeast mannitol agar at 26°–28°	Colonies of *Rhizobium* usually absorb very little of the dye and so remain practically colourless or only slightly pink (*R. meliloti*)	Marked absorption of the dye
3. Growth and change of pH on peptone glucose agar, 2 days at 30°	*Rhizobium* grows poorly on this medium and causes little change of pH	Abundant growth and marked pH change
4. Changes in litmus milk at 26°	Slow or no change towards acid or alkaline reactions; clear ("serum") zone sometimes formed	Rapid growth and change
C. *Invasiveness*	Nodulation of a legume under bacteriologically controlled conditions. See Table 2 for species recognition	
D. *Serology*	Agglutination (flagellar and/or somatic) with *Rhizobium* antisera, at 1/200 dilution or below is strong presumptive evidence of *Rhizobium* and some indication of *Rhizobium* species. Note, however, cross-reactions between *Agrobacterium* and *R. meliloti*	
E. *Bacteriophage sensitivity*	Similar specifications and limitations as (D)	

*For definition of *Rhizobium* species see Table 1.

Serological differences

The serological reactions of *Rhizobium* strains can be studied by using agglutination, complement fixation and precipitation reactions, but the agglutination reaction is most used because of its simplicity (Purchase, Vincent and Ward, 1951; Koontz and Faber, 1961; Graham, 1963*b*; Škrdleta, 1965; Drozanska, 1966; Holland, 1966). Table 3 shows examples of agglutination in which two antisera to strains of *R. trifolii* were tested against strains of *R. trifolii*, *R. phaseolus*, *R. leguminosarum* and *R. lupini*. Although the use of serological tests for species or strain identification is limited by cross-reactions and autoagglutination, they can be of great value in recognizing strains of rhizobia in nodules of field plants (Thornton and Kleczkowski, 1950; Read, 1953; Jenkins, Vincent and Waters, 1954; Means, Johnson and Date, 1964). Strain specificity depends largely on the separate recognition of flagellar and somatic agglutination, and requires the use of partially absorbed sera (Vincent, 1941; 1942; Purchase, Vincent and Ward, 1951). The gel immune-diffusion technique seems a convenient method for studying somatic antigens for strain differentation (Dudman, 1964).

Bacteriophage susceptibility

Strains of *Rhizobium* differ in their susceptibility to bacteriophage (Oshima, 1953; Kleczkowska, 1957) and some are lysogenic (Marshall, 1956; Takahashi and Quadling, 1961; Schwinghamer and Reinhardt, 1963; Kowalski, 1966). As with the serological reactions, differences are not yet sufficiently specific to be used to identify strains. Roslycky, Allen and McCoy (1960) report lysis of rhizobia by bacteriophages of *Agrobacterium*.

Modern Views on Classification

The taxonomy of *Rhizobium* presents special problems because of its dependence on symbiotic properties. Initially, only one species was recognized, *R. leguminosarum* (formerly called *Bacillus radicicola*), but as the complex patterns of cross-infectibility were explored more species were proposed. However, it soon became clear that infection specificity alone was an inadequate basis for speciation and only the six species given above (Table 1) were accepted.

Graham (1964) studied 83 strains of rhizobia and 38 strains of several other genera, including *Agrobacterium*, by comparing many of their morphological and physiological characters. Computer analysis of the results (without weighting characters) indicated three major groupings. All strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* (except one) were

TABLE 3. Species allocation based on agglutination reactions

Test organism	Agglutination with Antisera against:						
	R. leguminosarum	*R. phaseoli*	*R. trifolii*	*R. lupini*	*R. japonicum*	Cowpea rhizobia	*R. meliloti*
R. leguminosarum	+	±	±	—	—	—	—
R. phaseoli	±	+	±	—	—	—	—
R. trifolii	±	±	+	—	—	—	—
R. lupini	—	—	—	+	±	—	—
R. japonicum	—	—	—	±	+	—	—
Cowpea rhizobia	—	—	—	±	±	—	—
R. meliloti	—	—	—	—	—	—	+

+ = Flagellar and/or somatic cross-reaction common; ± = flagellar and/or somatic cross-reaction uncommon; — = no flagellar or somatic cross-reaction.

grouped together and Graham suggested that this group should be consolidated as *R. leguminosarum* Frank (the type species in the Bergey classification). The second group of fast growing strains proposed consisted of *R. meliloti*, *A. radiobacter* and *A. tumefaciens*. These strains are closely related and probably should be regarded as a single species, though Graham was reluctant to do this because only few strains were tested. He therefore suggested the retention of *R. meliloti* and the consolidation of the *Agrobacterium* strains to form a new species, *R. radiobacter*. The third group comprises the slow-growing species *R. lupini*, *R. japonicum* and *Rhizobium* spp. (Bergey) and Graham tentatively suggests that this group should be separated at the generic level and given the new name of *Phytomyxa japonicum*.

DeLey and Rassel (1965) studied DNA base composition of *Rhizobium*. The fast growing peritrichous strains with a %(G+C) of 59·1–63·1 correspond to the *R. leguminosarum* and *R. meliloti* groups of Graham; and the slow-growing subpolarly-flagellated strains with a %(GÆC) of 62·8–65·5, correspond with *R. japonicum*.

Separation of the rhizobia into two main groups is also advocated by Norris (1956). He regards one of these groups as "ancestral"; adapted to infertile tropical soils and able to infect very many tropical host species. This group corresponds to the *R. japonicum* and *Rhizobium* spp. in the Bergey classification. The other group comprises strains adapted to fertile, calcium-rich soil more typical of temperate regions. Symbiosis is greatly restricted in this "calcicole" group which includes the other five Bergey species.

Most of the work on the legume–*Rhizobium* symbiosis has been done with agricultural legumes of the temperate regions, which are not representative of the Leguminoseae as a whole. This family comprises over 14,000 species, most of them tropical or subtropical, and many of them trees. Only about 1200 species have been examined for the presence of nodules and not all of these nodulate (Allen and Allen, 1961). Even among host species that are regarded as invariably forming nodules, such as *Trifolium pratense* and *Glycine max*, non-nodulating lines have been bred (Nutman, 1949; Clark, 1957). More needs to be known about the nodule bacteria of legumes generally before classification of *Rhizobium* can be rationalized and completed.

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The Identification of Yellow-Pigmented Rods

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The ability to produce yellow or orange pigments is widely distributed in bacteria. Since superficially similar colony colour may be due to the presence of different compounds, emphasis on this character in identification procedures is of dubious merit. Carotenoids have been described from twelve families of bacteria (Ciegler, 1965) and problems in using pigment production as a taxonomic criterion arise from the effects of variations in media and temperature, or growth in light or dark. Whilst chemical characterization of pigments may show species or generic specificity in certain cases e.g. *Xanthomonas* (Starr and Stephens, 1964), much greater knowledge is required before the use of such methods can be employed for routine bacterial identification.

Use of the generic name *Flavobacterium* (Bergey *et al.*, 1923) was accompanied initially by a description allowing virtually any yellow-pigmented rod to be ascribed to the genus, regardless of the extent to which other characteristics might suggest a relationship to better defined but normally non-pigmented groups. In the circumstances it is hardly surprising that the organisms assigned to this genus in the past appear heterogeneous. Subsequent revision of the generic definition (Breed, Murray and Smith, 1957) has limited it to Gram-negative rods but many of the current problems of identifying yellow-pigmented rods still centre on the inadequate characterization of the genus *Flavobacterium*. This problem is made more complex by the fact that the supposed re-isolate of the type species (Weeks, 1955) has since proved to be a *Cytophaga* (see discussion of latter). Therefore we believe that the approach to the identification of yellow-pigmented rods should centre on their possible relationship to the well defined genera before relegating them to *Flavobacterium*. The tables shown here include better defined genera and groups containing only a few yellow-pigmented representatives, as well as those which usually appear yellow or orange.

Test Methods

The following tests have proved most useful for generic and group differentiation.

Gram stain. Heat-fixed smears should be prepared from cultures of different ages and, wherever possible several media.

Oxidase. The method of Kovacs (1956) is used.

Phosphatase. Following the technique of Barber and Kuper (1951). Phenolphthalein diphosphate (0·01% w/v) is used, incorporated in any basal medium suitable for growth of the test organism. Incubation for 3–5 days should be allowed before exposure of the plates to ammonia vapour, because of the relatively slow growth of *Cytophaga* spp. etc., compared with *Staphylococcus* spp. for which the test was originally designed. Occasionally, colonies of some positive strains fail to change colour possibly due to masking by the normal yellow of the colony; a halo of mauve pink can be seen in the medium surrounding the colony in such cases.

Motility (flagellation). Electron microscopy is preferred to differentiate polar from peritrichous flagellation but a staining method may be successfully employed in some cases, such as a modification of the Casares–Gil technique (Gemmell and Hodgkiss, 1964). Motility examinations should be made from several media before an organism is recorded as non-motile, and, where initial examinations have been made on cultures incubated at 30–37°, a re-examination should be made at 20°.

Spreading growth. Plates of a medium containing 0·1% w/v yeast extract and 0·8% w/v Ionagar No. 2 (Oxoid), pH 7–7·4, sterilized by autoclaving at 121° for 15 min, are prepared. Surface moisture is dried from the plates before incubation. Typical spreading growth takes the form of a thin film extending rapidly over the surface from the site of inoculation; some cultures will cover the surface of a 9 cm diam. plate in a few days from a central inoculum. Delayed spreading occurs with some cultures, outgrowths suddenly appearing after incubating for a week or more. Some fruiting myxobacteria and cellulolytic *Cytophaga* spp. spread through the agar gel rather than on the surface.

Bacteriolytic activity. A coliform such as *Klebsiella aerogenes* is grown on nutrient agar for 24–48 h. The growth is suspended in sterile distilled water and washed twice by centrifuging in distilled water. The sediment is resuspended in sterile distilled water and spread over the surface of water agar plates (1·0% w/v Oxoid Ionagar No. 2). The surface of the plates is dried at 45°. One 10 ml agar slope of coliform culture should provide sufficient cells to prepare two plates. Test cultures are inoculated onto the plates as spots or streaks and incubated at 25°. The plates are examined at intervals up to 14 days for lytic zones extending from the inoculum.

Fruiting body formation. Plates used to test for bacteriolytic action should be examined periodically for the presence of fruiting bodies within the lytic zones. A hand lens or low-power microscopic examination should be employed although in some cultures the fruiting bodies may be visible to

the naked eye. Incubation for fruiting body formation should be extended up to a month. Prolonged incubation on 0·1% yeast extract agar occasionally leads to fruiting body formation but rich media appear to inhibit their production. For further information and photomicrographs of fruiting bodies the review by Kühlwein and Reichenbach (1965) should be consulted.

Microcysts. The microcysts of *Sporocytophaga myxococcoides* can be demonstrated in cultures on cellulose. The fruiting bodies of myxobacteria consist of aggregates of cells which in *Myxococcus* spp. and *Chondrococcus* spp. appear as microcysts, contrasting with the vegetative cells of the same culture in size and shape.

Sensitivity to Polymyxin B. Plates of a suitable medium for growth of the test culture are inoculated with a broth suspension by flooding the surface and then draining off excess liquid. A Multodisk (Oxoid) containing polymyxin B 100 units in one of the tips is placed on the plate. The plate is incubated and examined at times dependent on the rate of growth of the culture.

Oxidative-fermentative attack on carbohydrates. The medium and methods of Hugh and Leifson (1953) are satisfactory for genera such as *Pseudomonas*, *Aeromonas*, *Vibrio* and *Erwinia*. Demonstration of the fermentative action of certain species of *Cytophaga* requires special media such as those of Bachmann (1955) and Anderson and Ordal (1961).

GC ratios. The values shown have been taken from the literature. For reviews see Marmur, Falkow and Mandel (1963) and Hill (1966).

Identification

The major features for identifying the individual genera and groups are shown in Table 1. Since many yellow-pigmented rods are isolated from selective enrichments on cellulose, a second key, Table 2, shows the differential features of some genera to which the cellulolytic yellow-orange organisms may be assigned.

Gram-positive strains

Following the revised description of *Flavobacterium* (Breed *et al.*, 1957), all Gram-positive strains should be excluded from the genus. However, some workers (Ferrari and Zannini, 1958; Ferrari, 1963) continue to prefer placing both Gram-positive and Gram-negative strains in the same genus.

It is advisable to examine a series of preparations from cultures of different ages to establish the Gram reaction since many coryneform organisms are easily over-decolourized and on occasions may appear Gram-negative. Yellow-pigmented organisms are common amongst the

TABLE 1. Identification of Yellow-pigmented rods

	Gram	Oxidase	Phosphatase	Motility (flagellate)	Polar flagella	Spreading growth	Bacteriolytic	Fruiting bodies	Microcysts	Sensitive to Polymyxin B.	Carbohydrates: Oxidative	Carbohydrates: Fermentative	GC ratio (approximate range)
Cytophaga	—	+	+	—	—	×	—	—	—	—	×	×	30–40
Sporocytophaga	—	+	+	—	—	×	—	—	+	—	×	—	30–40
Fruiting myxobacteria	—	+	+	—	—	+	+	+	+	±	·	—	68–72
Pseudomonas	—	×	×	+	+	—	—	—	—	+	×	—	55–68
Xanthomonas	—	—	·	+	+	—	—	—	—	·	+	—	62–68
Cellvibrio	—	+	—	+	+	—	—	—	—	+	+	—	·
Vibrio/Aeromonas	—	+	·	+	+	—	—	—	—	·	—	+	{V 40–48 A 56–60
Coliforms (including *Erwinia lathyri* *Escherichia aurescens*)	—	—	×	×	—	—	—	—	—	·	—	+	50–56
? *Flavobacterium*	—	·	·	×	—	—	—	—	—	·	+	—	·
Coryneforms	+ or ×	—	×	×	·	—	—	—	—	·	×	×	> 48
"Pleiston A"	×	—	—	—	—	×	—	—	—	—	+	—	61

+ = Positive; — = negative; × = variable; · = unknown; ± = weak positive.

TABLE 2. Differentiation of some cellulolytic types

	Gram	Oxidase	Carbohydrates Oxidative	Carbohydrates Fermentative	Polymyxin B sensitive	Microcysts	Flagella
Cytophaga	−	+	+	−	−	−	−
Sporocytophaga	−	+	+	−	−	+	−
Cellvibrio	−	+	+	−	+	−	+
Cellulomonas	+	−	−	+	·	−	+

+=Positive; −=negative; ·=unknown.

coryneforms and have been described in the genera *Arthrobacter*, *Cellulomonas*, *Brevibacterium* and *Microbacterium*.

Pseudomonas, Xanthomonas, Cellvibrio

Some strains of oxidative, polarly flagellate, Gram-negative rods produce a non-diffusible yellow pigment. These are classifiable as pseudomonads and are usually sensitive to polymyxin B. Identification of *Pseudomonas* has been discussed by Hendrie and Shewan (1966) and Stanier, Palleroni and Doudoroff (1966), *Xanthomonas* properties and definition are given by Hayward (1966).

Cellvibrio. These may be regarded as cellulolytic pseudomonads; specific identification is made on the basis of pigmentation and growth on various carbohydrates.

Vibrio, *Aeromonas*. Occasional yellow-pigmented strains of Gram-negative, polarly flagellate, oxidase positive, fermentative rods may be encountered (e.g. *Vibrio marinus*). These should be considered as strains of *Vibrio* or *Aeromonas* and identified accordingly (Bain and Shewan, 1968).

Coliforms

Fermentative, oxidase negative and Gram-negative rods including *Erwinia* should be classified as coliforms. Species identification can be made from the keys of Carpenter, Lapage and Steel (1966) for most of the *Enterobacteriaceae*, whilst the properties of *Erwinia herbicola* have been discussed by Graham and Hodgkiss (1967). This organism appears to be widely distributed and may be considered to include, or is closely related to *Bacterium herbicola*, *Erwinia lathyri*, *E. ananas*, *E. milletiae*, *E. uredovera* and *Chromobacterium typhi-flavum*.

A number of organisms previously identified as *Flavobacterium* have the superficial biochemical characters of coliforms, including strains often associated with brewery yeasts, e.g. *Flavobacterium proteus*. The generic name *Obesumbacterium* has been proposed for *F. proteus* (Shimwell, 1963); the relationship of this species to the coliforms requires further investigation.

Cytophaga, Sporocytophaga *and Fruiting Myxobacteria*

The original concept of the genus *Cytophaga* was a group of cellulolytic bacteria with flexuous cells, gliding motility, and not easily stained. It was subsequently expanded to include nutritionally diverse forms from soil and water, and particularly the marine environment; many of these forms were easily stained by the ordinary bacteriological methods (Stanier, 1942, 1947).

The problem of differentiating *Cytophaga* from *Flavobacterium* arose when it was shown that an organism believed to be a re-isolate of *Flavobacterium aquatile*, type species of the genus, was more correctly regarded as a *Cytophaga* although neither flexuous cells nor gliding motility were demonstrable (Weeks, 1955; Colwell and Mandel, 1964; Follett and Webley, 1965). The phenomenon of gliding motility has been used (Soriano and Lewin, 1965) as a primary taxonomic criterion but there are many difficulties attached to the use of this feature for purposes of identification, particularly the development of unequivocal methods (Lautrop, 1965) for its demonstration. The apparent association of spreading growth with the group of organisms designated Pleiston A by Floodgate and Hayes (1963) led these authors to regard the group as consisting of *Cytophaga* strains. Subsequent examination of some of these strains has shown their GC ratios to be *c*. 61% (DeLey and Van Muylem, 1963), whilst the group which Floodgate and Hayes considered to be *Flavobacterium* (Pleiston G) can now be identified as *Cytophaga*.

One possible source of confusion with *Cytophaga* strains is the finding of weak and delayed oxidase reactions by some cultures when grown on sea water agar although originally isolated from the marine environment. The same strains grown on nutrient agar containing 0·5% salt have given unequivocal positive reactions.

Certain morphological features are often helpful in differentiating *Cytophaga* strains:

(a) Appearance of long, flexuous rods in very young cultures (C. Quadling, personal communication, 1966).

(b) Production of star shaped aggregates, e.g. *Cytophaga columnaris* (Garnjobst, 1945), "*Flavobacterium capsulatum*" (Leifson, 1962).

(c) Ovoid cells in pairs in older cultures (3–10 days). This is often most striking when examined by electron microscopy, the cells may have an irregular outline and usually extracellular slime (Fig. 1).

Two characteristics of *Cytophaga* shown in Table 1, phosphatase activity and resistance to polymyxin B, are also in agreement with those reported from a computer analysis of some soil strains by Quadling (personal communication, 1966).

At the present time, subdivision of *Cytophaga* can be made into the following groups.

1. Cellulolytic species, further differentiated by superficial pigmentation into *C. hutchinsonii* and *C. lutea*, yellow; *C. aurantiaca*, orange; *C. rubra*, pink; and *C. tenuissima*, olive green.
2. Chitinoclastic, *C. johnsonii*.
3. Agarolytic oxidative species such as *C. krzemieniewskae*, *C. diffluens* and *C. sensitiva*.

4. Fermentative species, such as *C. fermentans*, *C. succinicans* and *C. salmonicolor*.
Strains of *C. fermentans* and *C. salmonicolor* which attack agar have been described (Veldkamp, 1961).
Since many species currently known under the genus *Flavobacterium* are

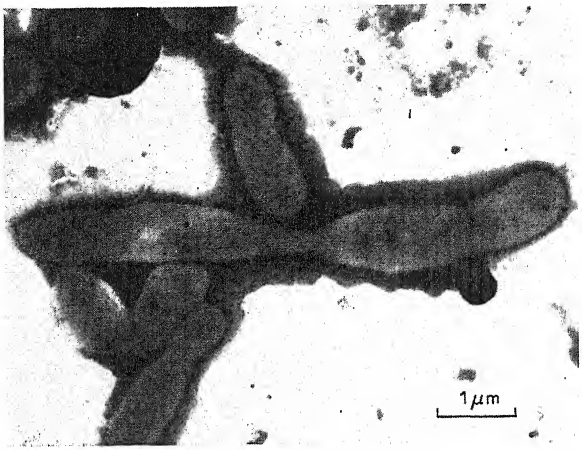

FIG. 1. *Cytophaga* sp. NCMB 1314, electron micrograph of phosphotungstic acid negative stained specimen.

undoubtedly more properly classified as *Cytophaga*, the range of known activities needs to be extended and will require considerable revision to permit species identification, and possibly the subdivision of the family Cytophagaceae into a number of genera including *Saprospira*, *Sporocytophaga* and *Flexibacter* (Soriano and Lewin, 1965).

Separation of *Sporocytophaga* from *Cytophaga* rests on the demonstration of microcysts. In the type species *S. myxococcoides*, the microcysts contrast sharply in shape with the vegetative cells (Fig. 2) and are abundantly formed on filter paper media. In the only other species we have examined, *S. cauliformis* (Gräf, 1962), the rounded forms appear similar to some of the pleomorphic forms occurring in older cultures of most strains of *Cytophaga* and their relationship to microcysts must remain in doubt. Rounded forms, apparently non-viable, were found by Veldkamp (1961) in some *Cytophaga* cultures, he did not consider them to be microcysts.

The true fruiting myxobacteria share a number of features with *Cytophaga* and *Sporocytophaga*, particularly in having flexuous cell walls, gliding motility and often staining poorly. Stanier (1942) proposed classifying *Sporocytophaga* with the myxococci rather than with *Cytophaga* and this separation has continued (Breed, *et al.*, 1957). Demonstration of very

different GC ratios for *Cytophaga* and fruiting myxobacteria but similar ones for *Cytophaga* and *Sporocytophaga* (Mandel and Leadbetter, 1965) suggest that the latter two genera should be grouped together. As the demonstration of fruiting body formation is slow and often somewhat difficult, other features for separating the fruiting forms would be useful.

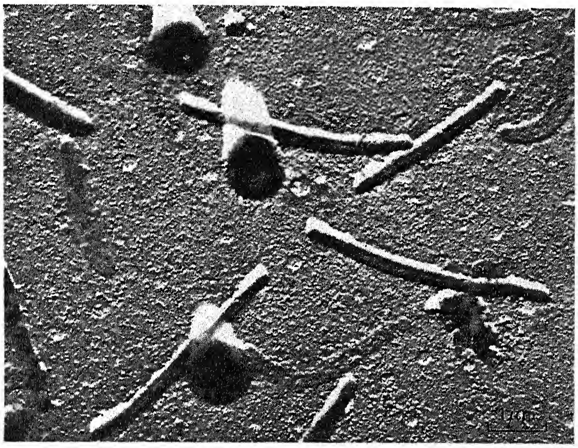

FIG. 2. *Sporocytophaga myxococcoides* NCIB 9470, electron micrograph, gold/palladium shadowed.

Our own studies have been limited to only a few strains of fruiting types. More extended observations may require a revision of the criteria which we would suggest at present, namely lytic action on coliform bacteria and weak sensitivity to 100 units of polymyxin B.

It is important to realize that many strains of both *Cytophaga* and fruiting myxobacteria grow well on media such as nutrient agar and blood agar, many appearing markedly haemolytic on the latter.

Flavobacterium

At the present time adequate means of defining the genus *Flavobacterium* are not possible because the re-isolated type species *F. aquatile* is now considered to be a *Cytophaga* sp. Also the strains of Pleiston G of Floodgate and Hayes (1963) which they called *Flavobacterium* are identifiable as *Cytophaga*. For convenience two subgroups of *Flavobacterium* may be considered.

(a) Non-motile rods, not identifiable as *Cytophaga*, Gram-negative, oxidative or not attacking sugars. Pleiston A of Floodgate and Hayes (1963)

may belong here although some strains appear to be Gram-variable and they also appear highly sensitive to penicillin.

(b) Gram-negative rods, motile by usually few peritrichous flagella, oxidative in sugars. Hayes (1963) examined 2 strains of this type and several others are known. The relationship of these strains to genera such as *Agrobacterium* or *Rhizobium* needs investigation, especially since one species of yellow-pigmented *Agrobacterium* is known (*A. gypsophilae*).

The genus *Empedobacter* was proposed by Brisou (1958) to encompass non-motile rods producing a yellow water-insoluble pigment, and could therefore be employed for (a) above. As most of the strains bearing this name which we have examined could be assigned to better defined genera it would appear that further work will be required before this nomenclature can be accepted.

We wish to thank Dr H. Reichenbach, Dr H. Voelz and Dr D. C. Gillespie for providing authentic cultures of fruiting myxobacteria used in our work, and Dr C. Quadling for providing unpublished observations on the *Cytophaga* group.

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Identification of *Aeromonas*, *Vibrio* and Related Organisms

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Great difficulty is generally encountered among systematists in defining accurately the genus *Vibrio* and in differentiating its members from the *Aeromonas* and *Pseudomonas* groups on the one hand and some of the Enterobacteriaceae on the other. Even the familial relationships are in some doubt, because some workers place the vibrios within the family Spirillaceae. We suggested some time ago that there was some evidence for grouping them in the Pseudomonadaceae (Shewan, Hobbs and Hodgkiss, 1960), but we see much merit in the more recent suggestions by Véron (1965) for the creation of a new family of Vibrionaceae; this to include both *Vibrio* and *Aeromonas* and possibly some rather less well-defined organisms such as *Plesiomonas* (Habs and Schubert, 1962).

The present determinative scheme is based on a detailed study over a number of years of the Gram-negative asporogenous rods generally classified as belonging to the *Pseudomonas*, *Aeromonas* and *Vibrio* groups.

The resultant differential criteria are based on a series of morphological and biochemical tests, amounting in all to approximately 150 features, using the definition of the latter term as given by Sneath (1957).

A few tests can be of great value in the primary differentiation, *viz.* motility; the oxidase test; attack on glucose; and the reaction to the vibriostatic agent 0/129 (2, 4-diamino-6, 7-di-*iso*propyl pteridine).

Motile, oxidase-negative, Gram-negative, fermentative rods should be examined for type of flagellation, since it is our experience that these are usually peritrichous and belong to the Enterobacteriaceae, while those which are oxidase positive, fermentative, polarly flagellated and insensitive to the compound 0/129 are normally *Aeromonas* spp.; we believe that species sensitive to 0/129 all belong to the *Vibrio* group. Moreover the former always give a positive reaction, and the latter, except the group pathogenic to poikilothermic animals, invariably a negative one in Thornley's (1960) arginine medium.

Although several workers have claimed that the use of the vibriostatic

agent is of doubtful value, this has not been our experience; and this will be fully discussed in a paper on the taxonomy of the *Vibrio* and allied species now in preparation.

Methods

For the key given in Fig. 1, the methods used are as follows:

Incubation

The usual temperature of incubation is 20°.

Gram reaction and morphology

Cultures are examined microscopically after 3 days' incubation on Oxoid Blood Agar Base (CM55).

Motility

This is determined by phase-contrast microscopic examination of the culture after 2 days' incubation in tryptone water (tryptone 1%, NaCl 0·5% in tap water).

Flagella

The arrangement of flagella is examined by electron microscopy.

Oxidase

The method of Kovacs (1956), using a platinum wire, is employed.

Dissimilation of glucose

The medium of Hugh and Leifson (1953) is used. If no growth is obtained, the test should be repeated with 3% NaCl incorporated into the medium.

Sensitivity to Vibriostatic agent 0/129

A plate of the CM55 agar is surface-seeded with the culture under test and the 0/129 applied in the form of a prepared disc instead of the saturated solution described by Shewan *et al.* (1954). Plates are incubated overnight at 20°. Whatman's antibiotic assay filter-paper discs (6 mm) are saturated in a 0·1% solution of 0/129, drained, and dried at 37°. The concentration of the solution is as recommended by Schubert (1962), who used chromatographic grade dioxan as solvent. This has been replaced in our laboratory with acetone (Houston 1966).

Action on carbohydrates

Tubes, of 1% (w/v) carbohydrate in peptone water with Andrades

indicator, containing Durham tubes, are used and examined after incubation periods of 1, 2, 4, 7 and 14 days.

Arginine

The method of Thornley (1960) is used.

Gelatin liquefaction

Stab cultures, in Oxoid nutrient broth No. 2 (CM67) + 12% gelatin, are incubated at 20° and examined after 1, 2, 4, 7 and 14 days.

NaCl tolerance

Plates of Oxoid CM55 agar with NaCl content of 7·5% are used.

Pigmentation

Production of a brown diffusible pigment is noted on Oxoid CM55 agar after 5 days. Formation of a non-diffusible orange-yellow pigment is noted after 5 days on sea-water agar (Evans peptone 1%; Oxoid Lab Lemco 1%; made up in a 3:1 mixture of aged sea-water and tap water.) Sterilized for 15 min at 121°.

Luminosity

Sea-water agar plates are inoculated and examined at daily intervals for up to 4 days in a totally blacked-out room. The examiner should remain 10 min in the dark before reading the plates.

Growth at 37°

A 3-day-old culture on CM55 agar is used.

Decarboxylases

The method of Møller (1955) is used.

Sensitivity to novobiocin

This is tested for by using Evans bacterial sensitivity test tablets (23, low potency), on the plates used to test the culture for sensitivity to the vibriostatic agent 0/129.

Spreading

This ability is noted on Oxoid CM55 or sea-water agar.

Response to Tests

The response of the various micro-organisms to the above tests is listed in Table 1 and the dichotomous key based on these tests is given in Fig. 1.

It will be noted from Table 1 and Fig. 1 that all the *Aeromonas* spp.

TABLE 1. Gram-negative, asporogenous, oxidase positive, fermentative rods

	Inhibition by 0/129	Gas from glucose	Motility	Large amount of brown diffusible pigment formed rapidly	Growth at 37°	Alkaline in Thornley's arginine medium	Lysine decarboxylase	Acid from inositol	Gelatin liquefaction	Poor/no growth on media with low salt content	Growth on media containing 7·5% NaCl	Spreading	Luminosity	Inhibition by novobiocin
Aeromonas spp.	—	+	+	—	+	+	— or [+]	—	+	—	—	—	—	—
A. salmonicida	—	+	—	+	—	+	×	—	+	—	—	—	—	—
A. formicans	—	—	+	—	+	+	—	—	+	—	—	—	—	—
C27 Organisms (*A. shigelloides*)	+	—	+	—	+	— or [+]	+	+	—	—	—	—	—	—
V. parahaemolyticus (*Oceanomonas* spp.)	+	—	+	—	+	—	+	—	+	—	+	×	—	×
Vibrio spp. pathogenic for man	+	—	+	—	+	—	+	—	+	—	—	—	—	+
Water vibrios (from India)	+	—	+	—	+	—	+	—	+	—	—	—	—	+
Vibrios pathogenic for poikilothermic animals	+	—	+	—	—	+	—	×	+	—	—	—	—	+
Luminous vibrios	+	—	+	—	—	—	[+]*	—	—	+	×	—	+*	+

+ = Positive; — = negative; × = variable; [+] = weak or transient positive; * = positive results obtained only when tested on media with added NaCl

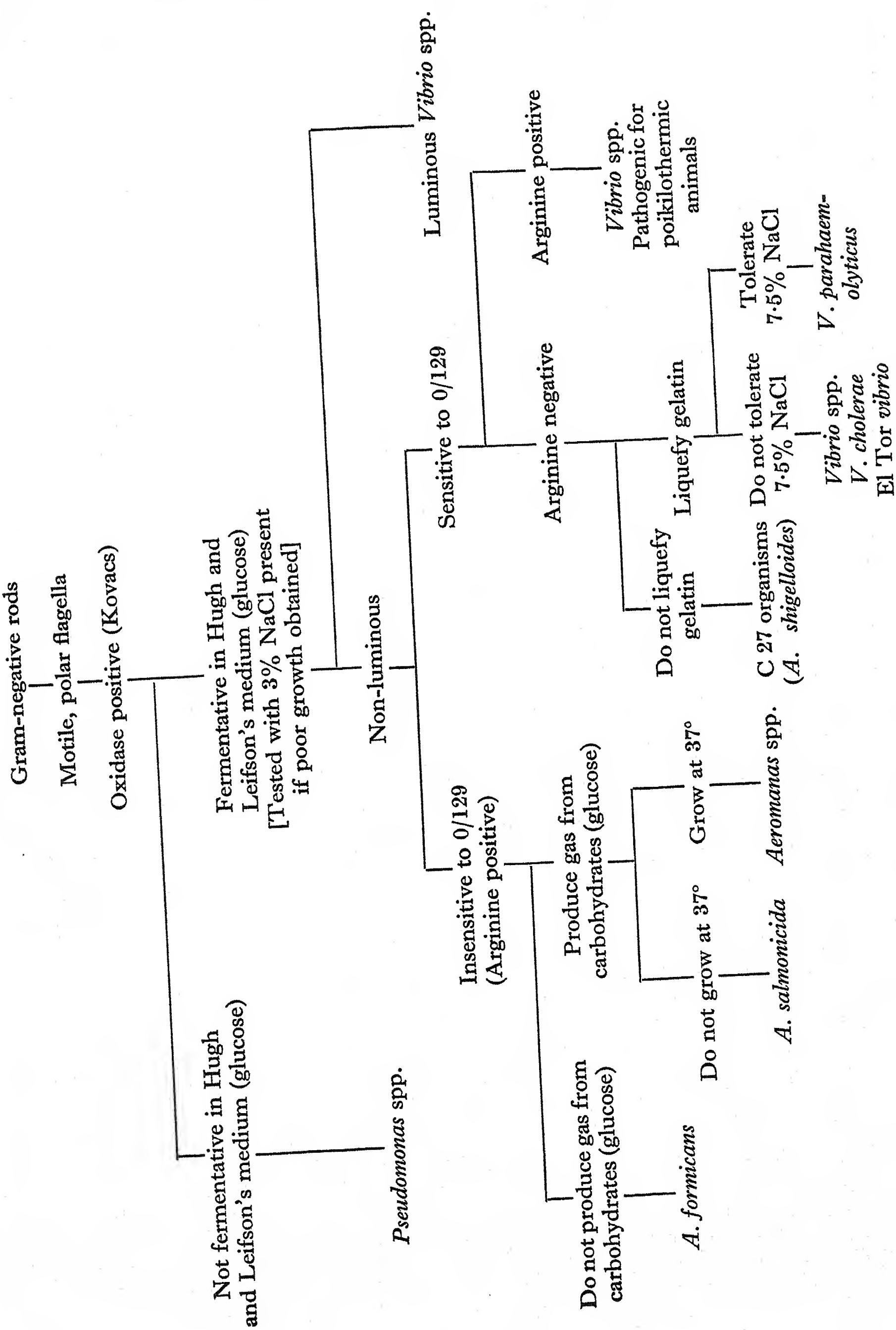
Gram-negative rods
Motile, polar flagella
Oxidase positive (Kovacs)
Fermentative in Hugh and Leifson's medium (glucose) [Tested with 3% NaCl present if poor growth obtained]
Not fermentative in Hugh and Leifson's medium (glucose)
Pseudomonas spp.
Luminous Vibrio spp.
Non-luminous
Sensitive to 0/129
Insensitive to 0/129 (Arginine positive)
Arginine positive
Vibrio spp. Pathogenic for poikilothermic animals
Arginine negative
Liquefy gelatin
Do not liquefy gelatin
Tolerate 7·5% NaCl
V. parahaemolyticus
Do not tolerate 7·5% NaCl
Vibrio spp. V. cholerae El Tor vibrio
C 27 organisms (A. shigelloides)
Produce gas from carbohydrates (glucose)
Grow at 37°
Aeromanas spp.
Do not grow at 37°
A. salmonicida
Do not produce gas from carbohydrates (glucose)
A. formicans

FIG. 1

including *A. formicans* and *A. salmonicida*, are insensitive to the vibriostatic agent, whereas the vibrios are sensitive.

Other strains sensitive to the vibriostatic agent are the C 27 species, (*A. shigelloides*), the luminous vibrios and *Vibrio parahaemolyticus*. It will also be noted that members of all these groups are arginine negative and lysine decarboxylase positive, tests which many workers believe to be important diagnostically in this field. (Ewing, Hugh and Johnson, 1961; Schubert, 1967; Thornley, 1960).

On the other hand, we have found that the "*Vibrio* spp." pathogenic for poikilothermic animals all give a positive arginine reaction and a negative lysine decarboxylase reaction. Their relationship to *V. cholerae* and *El Tor vibrio*, has still to be determined.

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The use of Cell Wall Analysis and Gel Electrophoresis for the Identification of Coryneform Bacteria

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Organisms of the family Corynebacteriaceae have one characteristic feature in common, i.e. "snapping division", which produces the V-forms seen in microscopic preparations. The differentiation of organisms within the family is not necessarily difficult using cultural methods, the main problem appears to be the establishment of criteria which will permit differentiation at the generic level. At present there are six genera. Of these, *Listeria* and *Erysipelothrix* appear relatively homogeneous, *Corynebacterium* and *Arthrobacter* are heterogeneous, *Cellulomonas* contains a number of species with very few differences, and doubts have been expressed as to whether *Microbacterium* should exist at all. The methods which have been used in this laboratory have been valuable not only for the identification of "coryneform" bacteria but have also helped to clarify some of the anomalies which exist among coryneform bacteria.

Details of the techniques of cell-wall analysis and starch gel electrophoresis are scattered throughout the literature and this Manual provides an excellent opportunity for collecting them into the one place.

Cell-wall analysis has undergone little change since it was first used by Cummins and Harris (1956) to investigate cell-wall composition in *Corynebacterium*; any changes which have been introduced have merely improved the clarity of the final result. Where other methods have been used they have given identical results when adequately purified cell-wall preparations have been used (Keddie, Leask and Grainger, 1966; Robinson, 1966*b*).

Without certain essential precautions the results obtained by different workers using starch gel electrophoresis may not be comparable. The methods to be described here were based on the original method of Smithies (1955), which was later modified by Baillie and Norris (1963) for the examination of bacterial extracts. These methods have since been used to investigate systematically the cell-free extracts of coryneform bacteria

(Robinson, 1966*a*, *b*) and other bacteria (Norris, 1964; Lund, 1965; Cann and Willox, 1965).

In the combination of these two techniques the amino acid composition of the cell-wall mucopeptide is used for primary differentiation and the patterns of enzymes produced by electrophoresis of the cell-free extracts in starch gel for identification of the organisms within the "cell-wall groups".

Methods

Disintegration

A dense suspension of washed cells in distilled water is disintegrated ultrasonically and the suspensions cooled during disintegration by immersion in ice-cold water. Disintegration is continued until few, if any, whole cells can be seen by phase-contrast microscopy or until there is a marked reduction in the opacity of the suspension.

The insoluble cell debris is harvested by centrifugation at 10–20,000*g* for 20 min and the supernatant removed and retained for electrophoresis.

Cell-wall analysis

Purification of cell walls

The crude cell wall deposit, resuspended in distilled water, is centrifuged first at 2000*g* for 10 min to remove whole cells and then at 10,000*g* for 15 min to harvest the cell walls.

Purification of the crude cell-wall fractions is carried out as follows:

(i) Digestion with 0·5% w/v potassium hydroxide in ethanol at 37° for 24–48 h. The alkaline ethanol is removed by washing several times in absolute ethanol and several times in distilled water.

(ii) Digestion in 0·05M phosphate buffer pH 7·6 containing 0·5 mg/ml each of ribonuclease and trypsin at 37° for 12–18 h.

(iii) Digestion in 0·02N hydrochloric acid containing 1 mg/ml of crystalline pepsin at 37° for 18–24 h.

The purified cell-wall mucopeptide is washed several times in distilled water, suspended in distilled water and stored at —22°.

Hydrolysis of cell walls

50 mg quantities (wet weight) of purified cell wall are hydrolysed in sealed tubes for 18–24 h at 105° in 6N hydrochloric acid. The hydrolysates are filtered and the hydrochloric acid removed by evaporating the filtrate to dryness *in vacuo* and redissolving the product in 0·25 ml of distilled water.

Separation and identification of amino acids

The following systems are used to detect the amino acids present in the hydrolysates.

(i) Paper electrophoresis on Whatmann 3MM paper in boric acid-sodium hydroxide buffer (boric acid 7·5 g, sodium hydroxide 3·75 g, distilled water to 1 l.) pH 10. Samples for electrophoresis are placed along an origin 8 cm from the cathode end of the paper. Electrophoresis is carried out by applying a potential of 9V/cm for 3h.

(ii) Paper electrophoresis on Whatmann 3MM paper in 0·025M sodium carbonate pH 11·5 (Perkins and Cummins, 1964) with the origin 1 cm from the cathode end of the paper. Electrophoresis is carried out by a potential of 9V/cm for $2\frac{1}{2}$ h.

(iii) Two-dimensional ascending paper chromatography on Whatmann No. 1 paper (10 in. square) using as solvents:
1st dimension—butanol: acetic acid: water (60:15:25);
2nd dimension—phenol: water (4:1) containing 0·25% of 33% ammonia.

(iv) One-dimensional descending chromatography on Whatmann No. 1 paper ($18\frac{1}{4}$ in. × $22\frac{1}{2}$ in.) for 15–18 h using as solvent methanol: pyridine:water:hydrochloric acid (96:12:21:3) (Rhuland, Work, Denman and Hoare, 1955).

Papers used for electrophoresis are dried in a hot air oven and the amino acids located by dipping the papers in 0·2% w/v ninhydrin in 5% v/v acetic acid in acetone. The solvent is removed from paper chomatograms with a warm or cold air draught and the amino acids located by dipping in 0·2% w/v ninhydrin in acetone. In both cases location is completed by heating the dried papers at 100° for 2–3 min.

The amino acids are identified from their Rf values, the colour of the spot obtained with ninhydrin and by the comparison of electrophoretic mobilities with those of pure compounds.

Starch gel electrophoresis

Preparation of cell-free extracts

Cell-free extracts are obtained by removal of the supernatant obtained after disintegration and centifugation. The clarified extracts are standardized, dispensed in ampoules and stored at −22°.

Standardization of extracts

The concentration of the cell-free extracts is measured turbidimetrically by the following method.

A portion of the extract is diluted to 4 ml with distilled water and inverted twice to mix: 6 ml of 5% w/v trichloroacetic acid is added (mix twice by inversion). The turbidity of the suspension is measured in an EEL Nephelometer (Evans Electro Selenium Ltd, Halstead, Essex) and the protein concentration of the extract calculated from a calibration curve prepared by measuring the turbidity of solutions containing known concentrations of ovalbumin (Koch-Light Laboratories Ltd, Colnbrook, Bucks) (Stadtman, Novelli and Lipmann, 1951).

Each extract is adjusted to give a final protein concentration equivalent to 20–30 mg ovalbumin per ml.

Concentration of the extracts, when necessary, is carried out either by collodion filter (Membranfiltergesellschaft, Göttingen, Germany) or by the addition of small quantities of Sephadex G-10 (Pharmacia Ltd, Uppsala, Sweden).

Preparation of Starch gels

The method of preparing starch gels is that used by Baillie and Norris (1963). Specially hydrolysed starch (Connaught Laboratories Ltd)* is heated gently in tris-citrate buffer (tris 2·295 g, citric acid 0·709 g, distilled water to 1 l.), pH 8·6. Heating is continued until the mixture after first becoming extremely viscous becomes more fluid; a vacuum is applied until the mixture boils freely and when this is released the mixture collapses to form a clear gel which is poured into casting trays (6 in. × 4 in. × $\frac{1}{4}$ in.) previously coated with silicone release agent, covered with a sheet of Melinex "O" film (ICI Ltd) and allowed to age for not less than 2 h. Although the concentration of starch used originally was the optimal concentration given by the manufacturers, subsequent batches of starch used at the optimum concentration have not produced the same results. The concentration of starch now used is that which is required to obtain established Ef values of the esterases and catalases of a control extract (Robinson, 1966*b*).

Electrophoresis

After ageing each gel is cut across its width 1 in. from one end and small strips of Whatmann 3MM paper (6 mm square) moistened with the sample, sandwiched between the cut faces of the gel. The samples are electrophoresed at 10 V/cm of gel, measured between the ends of the lint wicks used to connect the gel to the outer compartments of the electrophoresis tank. The sample origin is placed nearest the cathode and borate buffer (boric acid 18·5 g, sodium hydroxide 3·3 g, distilled water to 1 l.) pH 8·6 is used as tank buffer. During electrophoresis a brown line develops in the gel

* Obtained from Arnold R. Horwell, Cricklewood, London.

at the interface between the two buffer systems and electrophoresis is continued until this line has travelled 10 cm past the origin.

Detection of enzymes

At the end of electrophoresis each gel is cut into four horizontal slices, the upper and lower slices are discarded and the remainder stained for either esterase or catalase and peroxidase.

Esterase (Lawrence, Melnick and Weimer, 1960). The gel slice is flooded with 0·1M tris-maleate buffer pH 6·4 containing 2 ml 1% w/v α-naphthyl acetate in 50% acetone and 100 mg % w/v Fast Blue B salt. Staining is allowed to take place overnight (although active esterases can be detected in 2 h) when esterases develop as red bands.

Catalase and peroxidase (Robinson, 1966*a*). The gel slice is immersed in 2% w/v potassium iodide in 5% v/v acetic acid for 30–60 sec, washed thoroughly in running water and then immersed in 3% v/v hydrogen peroxide (100 vol.). Catalases appear as discrete white bands and peroxidases as intense blue bands against the blue background of the gel.

Recording of results

A control extract prepared from a strain of *Microbacterium lacticum*, National Collection of Industrial Bacteria (NCIB) 9919, is electrophoresed with each gel and the mobilities (Ef) of each of the enzyme bands of each extract determined relative to those in the control. It is emphasized that the Ef is not an absolute value (*q.v.* Rf in chromatography) so that relationship with the control is absolutely essential if the results obtained on different occasions and with different, but closely related, organisms are to be comparable.

The results obtained are recorded diagrammatically and also photographically on 35 mm Ilford Microneg Pan film.

Separation of Coryneform Bacteria into Cell-Wall Groups

FIG. 1. The separation of coryneforms by cell wall amino acid combination.

I *Alanine, Glutamic Acid,* DL-*Diaminopimelic Acid*
II *Alanine, Glutamic Acid, Glycine, Ornithine*
III *Alanine, Glutamic Acid, Glycine, Aspartic Acid, Lysine*
IV *Alanine, Glutamic Acid, Glycine, Aspartic Acid, Ornithine*
V *Alanine, Glutamic Acid, Lysine*
VI *Alanine, Glutamic Acid,* LL-*Diaminopimelic Acid*
VII *Alanine, Glutamic Acid, Leucine, Aspartic Acid,* DL-*Diaminopimelic Acid*
VIII *Alanine, Glutamic Acid, Glycine, Aspartic Acid, Component "U", Lysine*
IX *Alanine, Glycine,* 2:4 *Diaminobutyric Acid*

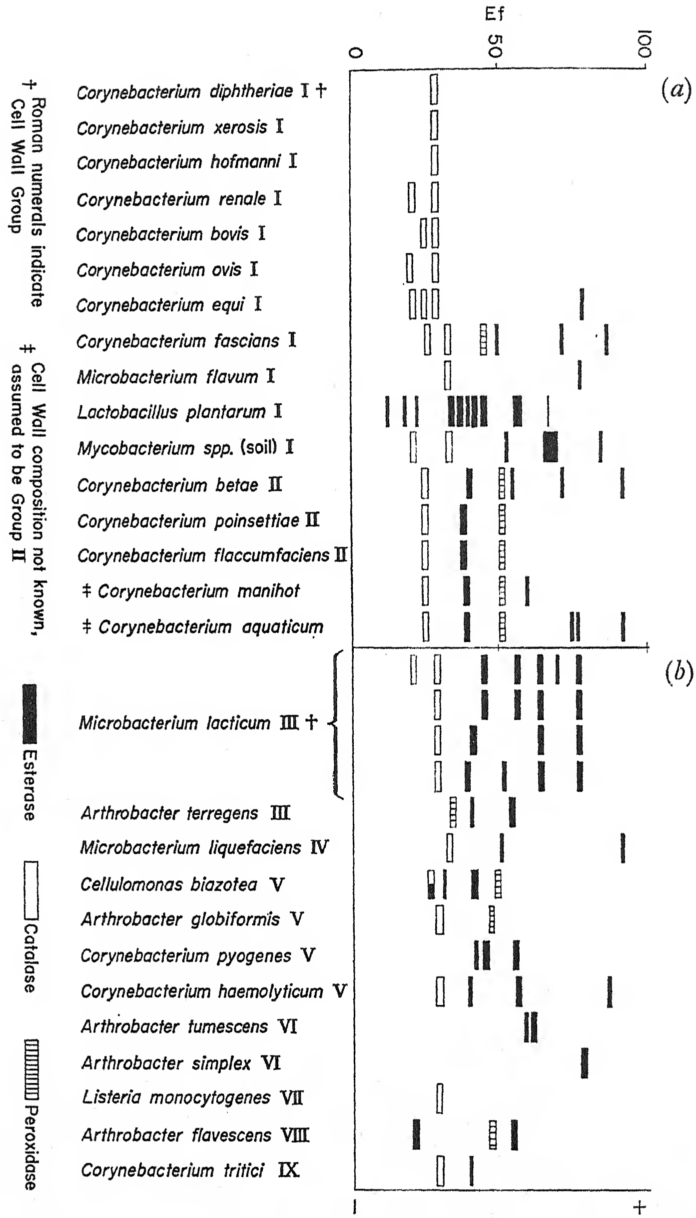

FIG. 2. The esterase, catalase and peroxidase patterns obtained by gel electrophoresis of the cell-free extracts of the coryneform bacteria of cell wall groups (*a*) I and II and (*b*) III—IX.

The separation of coryneform bacteria into cell-wall groups is shown in Fig. 1. The cell-wall composition of a number of organisms have been determined by the author and where no results have been determined reference has been made to the literature (Cummins and Harris, 1956; Keeler and Gray, 1960; Cummins, 1962; Keddie, Leask and Grainger, 1966). Nine combinations of amino acids have so far been found among coryneform bacteria. The occurrence of *Arthrobacter* in four of these groups emphasizes its heterogeneity.

The identity of the organisms within each of the cell-wall subgroups may be determined from the type and mobility of their enzymes. The enzyme patterns which have so far been established are shown in Fig. 2 (*a*, *b*).

The Ef values shown in Fig. 2 are slower than those given previously (Robinson, 1966*a*). At the time of writing the precise effect of modifications in experimental conditions is not known. When these effects are known it may be necessary to modify the Ef values of the enzymes of the control organism *Microbacterum lacticum* NCIB 9919. Meanwhile, the enzyme pattern of the different organisms presented here are sufficiently distinct to permit their separation despite the absence of accurate Ef values.

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The Genus *Bacillus*: Aids to the Identification of its Species

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Prior to 1946 the differentiation of species within the genus *Bacillus* and the identification of many of its members was extremely difficult. This situation changed with the publication of the determinative key of Smith, Gordon and Clark (1946). This was subsequently enlarged in the 1952 monograph (Smith *et al.*, 1952) and further revised by Smith and Gordon in the current edition (7th) of *Bergey's Manual*, (Breed, Murray and Smith, 1957). Reference to the earlier scheme of Smith and Clark (1937) bears witness to the substantial advances made during the subsequent decade. Some of this rapid progress would have been impossible without the basic contributions of Gibson (1934*a*, *b*, 1937, 1944) dealing with the characterization of *B. pasteurii*, *B. subtilis* and *B. licheniformis*. Some of the essential features of the later American scheme can also be seen in the keys of Gibson (1937) and Gibson and Topping (1938), e.g. the swelling of the sporangium as a group feature, and size and vacuolation as interspecific characters.

Experience over the past fifteen years has testified to the soundness of the scheme of Smith *et al.* (1952), and its validity has been further substantiated by recent studies of additional criteria, e.g. nutrition (Knight and Proom, 1950; Proom and Knight, 1950, 1955), spore surface morphology (Bradley and Franklin, 1958), analysis of amino-acid pools (Jayne-Williams and Cheeseman, 1960), and antigen distribution (Norris and Wolf, 1961). Even Adansonian classification by computer analysis has confirmed many of the affinities indicated by Smith *et al.* (1952), although at the same time some anomalies have become apparent (Sneath, 1965). Thus, in spite of some inherent difficulties, the scheme has proved most valuable; these difficulties are discussed later.

Definition of the genus

The following definition of the genus is suggested: "Rod-shaped organisms which are spore bearing, usually Gram-positive, catalase producing and capable of sporulating aerobically".

The latter characteristic has been included to distinguish the members

of the genus from some aerotolerant clostridia, which have the ability to produce catalase, but fail to sporulate under aerobic conditions (Hall and Duffett, 1935).

Non-sporulating variants have been described in several of the species. Since biochemical reactions alone are frequently inadequate for the identification of such strains, a supplementary study of their vegetative cell antigens may be required. By means of the latter criterion, Baillie (1967) was able to demonstrate the relationship (virtual identity) between an asporogenic variant of *B. cereus* and its spore-forming parent type. In this connection the esterase patterns of the vegatative phase may also supply an additional criterion. On present evidence it is doubtful whether bacteriophage patterns could be used at a similar specific level.

Serology

Although its full applications await a detailed study, existing evidence strongly suggests that the serology of *Bacillus* could contribute substantially towards a more fundamental definition of the units within the genus. Thus, the different antigens of an organism (flagellar, somatic or spore) operate at different levels of specificity. Where interest is centred on the identification of particular strains, or biotypes, flagellar material provides the antigen of choice (Davies, 1951; de Barjac and Bonnefoi, 1962) and for broader subdivision within a species, somatic antigens could be usefully exploited (Norris and Wolf, 1961). For identification at species level the use of spore antigens would appear a most promising development. This is illustrated in Table 1. It will be seen that, whereas spore antigens (agglutinogens) virtually demonstrate species specificity, the same cannot be said for the vegetative antigens (somatic "O" agglutinogens). In the case of the latter, cross reactions occur between organisms showing markedly different morphological and physiological characteristics e.g. *B. licheniformis* and *B. alvei.*

Concerning spore antigens, it is of some interest to observe that agglutinogens and precipitinogens may differ in their taxonomic significance. In group I (Tables 3 and 4), for example, there are strong indications that in the small-celled species the two types of antigen give identical reactions, and either is indicative of the species (Table 2). In the large-celled species, however, i.e. *B. cereus* or *B. megaterium*, the agglutinogens are indicative of intraspecies groupings; the precipitinogens, however, retain their species rank. *The preparation of an antiserum to the spores of a single strain could be used therefore for the identification of any strain with that species.* Table 2 also shows that differences in the specificity of spore agglutinogens and precipitinogens occur in group II.

TABLE 1. Interspecific agglutination reactions of vegetative and spore antigens (modified from Norris and Wolf, 1961)

Antiserum prepared against	Antigen: *B. cereus* M.8	*B. megaterium* 2529	*B. subtilis* 3610	*B. pumilus* 2746	*B. licheniformis* 2843	*B. coagulans* K & P	*B. polymyxa* 2002	*B. circulans* 2925	*B. alvei* 2198	*B. brevis* 2934	*B. laterosporus* 68A	*B. sphaericus* 2546
B. cereus M8	+	—	—	—	—	—	—	—	—	—	—	—
B. megaterium 2529	—	+	—	*	—	—	—	—	—	—	—	—
B. subtilis 3610	—	—	+	+	—	—	—	—	—	—	—	—
B. pumilus 2746	—	—	†(tr)	+	—	—	—	—	—	—	—	—
B. licheniformis 2843	—	—	—	—	+	—	—	—	*	—	*	—
B. coagulans K & P	—	—	—	—	—	+	—	—	—	*	*	—
B. polymyxa 2002	—	—	—	—	—	—	+	—	—	—	—	—
B. circulans 2925	—	—	—	—	—	—	—	+	—	—	—	—
B. alvei 2198	—	—	—	—	*	—	—	—	+	—	—	†(tr)
B. brevis 2934	—	—	—	—	—	*	—	—	—	+	*	—
B. laterosporus 68A	—	—	—	—	*	—	—	—	—	*	+	—
B. sphaericus 2546	—	—	—	—	—	—	—	—	†(tr)	—	—	+

\+ Positive reactions of the spore *or* vegetative cell antigen with its homologous antiserum.
* Additional reactions between vegetative cell antigens and heterologous vegetative cell antisera.
† Additional reactions between spore antigens and heterologous spore antisera.
— Negative reaction.
(tr) Trace reaction only at 1/20 titre.

TABLE 2. Results of tests in six species which illustrate the specificity of the spore precipitin and agglutinin when tested against spore antigens of other strains of the same species (Adapted from Norris and Wolf, 1961)

		Antiserum prepared against					
		Group I				Group II	
		B. cereus	*B. megaterium*	*B. licheniformis*	*B. subtilis*	*B. polymyxa*	*B. brevis*
Number of strains positive/ number tested	Agglutination	7/27	2/7	18/18	25/25	39/39*	1/4
	Precipitation	27/27	7/7	17/18	25/25	Not tested	4/4

* Davies, 1951

✓ Subdivision within the Genus

In the scheme of Smith *et al.* (1952) the subdivision into three groups on the basis of spore shape and sporangial swelling has its limitations. This is particularly true in distinguishing oval from spherical spores, i.e. their groups 2 and 3. Demarcation between groups 1 and 2 is less difficult.

During the course of our work with these organisms, extending over several years, many difficulties have been encountered and these have led us to try out a number of alternative identification schemes. Difficulties arising from the presence of both oval and spherical spores in purified cultures of *B. panthothenticus*, the occurrence of strains of aerobacilli (*B. macerans* and *B. polymyxa*) giving rise to spherical spores and the occurrence of strains of *B. coagulans* with and without swollen sporangia, have led us to formulate the particular version presented here (Tables 3–6). It should be emphasized that, whilst it has been largely based on the facts from Smith *et al.* (1952), the responsibility for the rearrangement of the key is ours.

The opportunity has been taken to emphasize in the definition of group III its tendency to biochemical inertness and as a result *B. pantothenticus*

has been transferred to group II, where it more logically belongs. By the same token of biochemical activity, atypical aerobacilli (spherical spores) no longer constitute a problem and are readily retainable in group II. On the other hand, the two distinct sporangial types of *B. coagulans*, which probably represent distinct species, have been provisionally dealt with by including the species in each of the two groups (I and II).

Extensive studies with the thermophilic species have persuaded us of the constancy and importance of their physiological characteristics. These have received due emphasis in the scheme. Indeed, preference has been given to physiological and nutritional requirements in all groups and thus the need for direct observation of size or vacuolation has been relegated to a confirmatory level. Nevertheless it must be recognized that within each species variant strains occur which, although otherwise identical, differ in respect of the particular characteristic selected for use in the key. The more important of these differences have been noted, e.g. the existence of lecithinase-negative strains in *B. cereus*, amino-acid-dependent strains in *B. subtilis* and anaerogenic variants in *B. polymyxa* and *B. macerans*.

TABLE 3

Gram-positive to Gram variable rods, sporulating aerobically and producing catalase

- Spores oval or cylindrical
  - Sporangia not definitely swollen; Spore wall thin — **Group I**
  - Spores usually oval[3]; Sporangia distinctly swollen; Spore wall thick — **Group II**
- Spores spherical; Sporangia swollen[1]; Non-fermentative[2]; Casein and starch not hydrolysed[2]; Nutritional requirements complex — **Group III**

[1] Some strains give rise to spherical spores in an unswollen sporangium.
[2] Strains giving positive results in these tests are treated under group II.
[3] Included here are some strains which have spherical spores but which are biochemically active (e.g. fermentative and caseolytic).

The three groups, their species and strains

Group I (*Table* 4)

This group comprises a series of well-defined species. The occurrence of types intermediate between species is much less marked than in group II.

TABLE 4

Spores oval or cylindrical. Sporangia not definitely swollen. Spore wall thin

Group I

- No growth at 60°, pH 6·0
  - No significant growth in glucose broth under anaerobic conditions
    - No acid in ammonium glucose salts medium
Glucose retardatory
      - Urease +ve
Casein not hydrolysed
B. lentus
      - Urease —ve
Casein hydrolysed
B. firmus
    - Acid in ammonium/glucose/ salts medium
Glucose non-inhibitory
      - V.P.—ve
Large cells (> 0·9 μm)
Vacuolated
B. megaterium
      - V.P. +ve
Small cells (< 0·9 μm)
Non-vacuolated
B. subtilis and variants[4]
  - Good growth in glucose broth under anaerobic conditions
    - Lecithinase —ve
Small cells (< 0·9 μm)
Non-vacuolated
B. licheniformis
    - Lecithinase +ve[2]
Large cells (> 0·9 μm)
Vacuolated
B. cereus and variants[3]
- Growth at 60°, pH 6·0[1]
B. coagulans

[1] Except for a few strains. Another characteristic of this species is its acidophilic nature.

[2] One or two variants are lecithinase negative.

[3] Strains of *B. cereus* var. *thuringiensis* are distinguished by crystal formation within the sporangium (Table 9) and strains of var. *mycoides* by their rhizoidal colony form. Characters differentiating *B. cereus* var. *anthracis* are detailed in Table 8.

[4] Recent genetic studies indicate the existence of non-typical amino-acid-dependent strains. *B. pumilus* has been treated as a variant of *B. subtilis*.

TABLE 5

Spores usually oval, rarely cylindrical or spherical. Sporangia distinctly swollen. Spore wall thick

Group II

No growth at 65°, pH 7·0	Growth at 65°, pH 7·0 *B. stearothermophilus*
No growth at 60°, pH 6·0	Growth at 60°, pH 6·0[1] *B. coagulans*
Spore not laterally displaced in the sporangium	Spore laterally displaced in the sporangium *B. laterosporus*
Do not produce gas from sugars	Produce acid and gas from sugars
	V.P. +ve *B. polymyxa*[2]
	V.P. −ve *B. macerans*[2]
Not pathogenic to insects Good growth on nutrient agar	Pathogenic to insects[3] Poor or no growth on nutrient agar *B. larvae* *B. popilliae* *B. lentimorbus*
Starch hydrolysed	Starch not hydrolysed
	No growth in glucose broth under anaerobic conditions *B. brevis*
	Growth in glucose broth under anaerobic conditions *B. pulvifaciens*
No growth in 10% salt broth	Growth in 10% salt broth *B. pantothenticus*
V.P. −ve, Indole −ve Arabinose and xylose fermented *B. circulans* complex	V.P. +ve, Indole +ve Arabinose and xylose not fermented *B. alvei*

[1] Except for a few strains. Another characteristic of this species is its acidophilic nature.

[2] Anaerogenic strains of *B. polymyxa* and *B. macerans* can generally be recognized by their ability to grow on a medium containing NH_4 as the sole nitrogen source when supplemented by biotin and thiamine.

[3] *B. larvae* is the cause of American foulbrood of honey bees and *B. popilliae* and B *lentimorbus* are the cause of milky diseases of the Japanese beetle.

TABLE 6

Spores spherical, sporangia swollen, non-fermentative. Casein and starch not hydrolysed. Nutritional requirements complex

Group III

NH_3, urea and alkaline conditions are not required for growth Usually grow on nutrient agar at pH 6·0[2] *B. sphaericus*	Require urea *or* NH_3 and alkaline conditions for growth[1] No growth on nutrient agar at pH 6·0 *B. pasteurii*

[1] The critical concentrations of these may vary with the strain.
[2] Some well-recognized varieties will only grow at a higher pH, e.g. 7·5.

Characterization of group I depends on a combination of morphological features, e.g. ellipsoidal to cylindrical spores, slight or no swelling of the sporangium and a tendency for the spores to be central rather than terminal. Reliance on a single character may present difficulties, e.g. many strains of *B. subtilis* show swelling of their sporangia.

Species and strains

B. coagulans

Smith *et al.* (1952) expressed reservations concerning this species. Their observation of two distinct types of sporangia made it difficult to decide to which of their first two groups the organism should be assigned. Its allocation to group I was largely arbitrary and was based mainly on physiological reactions. An examination of numerous strains has indicated that *B. coagulans* exists in a variety of morphological types (Wolf, unpublished data). In addition to the typical group I sporangial form (non-swollen), distinctly swollen sporangia also occur; the spores of either form may be oval or cylindrical. The size of the vegetative cell is often large and its width is 0·9 μm or even greater. The species is readily separable into two major and at least four subsidiary types. The principal reactions of the two main types are given in Table 7. Pending final resolution into distinct species the organism has been provisionally included in both groups I and II. The acidophilic and thermophilic properties of the species are important characteristics. Virtually all types grow at 60° and are capable of initiating growth at pH 5·3.

TABLE 7. *B. coagulans*. An examination of 130 cultures, which included all the eighty-four strains of Smith, Gordon and Clark (1952), showed the existence of two distinct types separable by physiological tests.* Some intermediate types were also apparent (Wolf, unpublished results)

	Growth					Fermentations in broth			
	Nutrient broth			Nutrient agar slope					
Type	pH 4·5	pH 7·7	+4% NaCl† 53°	pH 6·2 65°	pH 6·2 23°	V.P.	Litmus milk	Mannitol	Arabinose Sucrose Starch
A	—	—	—	+	—	—	Alk.	+	—
B	+	+	+	—	+	+	A.C.R.	—	+

Alk. = alkaline reaction.
A.C.R. = acid, clot and reduction.

* Incubation was in a water bath at 45° unless otherwise stated.
† Final concentration in medium.

B. cereus and its variants

It has been convenient to follow the precedent of Smith *et al.* (1952) in treating the well-recognized entities such as *B. anthracis* and *B. thuringiensis* as variants of *B. cereus*. We are aware of the equally strong case for treating these as distinct species (e.g. Heimpel, 1967). The importance of anthrax as a human and animal disease has been recognized by the inclusion of a table listing a number of *in vitro* tests which enable the differentiation of *B. cereus* var. *anthracis* from *B. cereus* (Table 8).

TABLE 8. Differentiation of *B. cereus* var. *anthracis* from *B. cereus* (Based on Burdon, 1956)

	B. cereus var. *anthracis*	*B. cereus*
Motility	—	+ (some —)
Salicin fermentation	—	+ (some —)
Sensitivity to penicillin (10 i.u./ml.)	+	—
Haemolysis of sheep blood	— or slow	+
Reduction of methylene blue	— or slow	+
Growth at 45°	— or slow	+
Capsulation and smooth colonies on HCO_3 media	+	—
Capsulation in vivo	+	—
Need for thiamine*	+	—

*Proom and Knight (1955).

The extensive research in recent years into the application of crystalliferous strains of *B. cereus* (i.e. var. *thuringiensis*) as insect pathogens has suggested the inclusion of a table which clearly distinguishes between the various types so far recognized (Table 9). The occurrence of lecithinase negative strains should be noted.

B. licheniformis

This organism has shown remarkable constancy in its characteristics and has presented no difficulties.

B. subtilis and its variants

Since pigmentation is a variable characteristic, the pigmented varieties of this species have not been listed separately. In most other respects these organisms show the typical reactions of the species.

B. pumilus

Whilst Gibson (1944) has expressed doubts about the need to distinguish this species from *B. subtilis*, Hanáková-Bauerová, Kocur and Martinec

TABLE 9. Classification of *B. thuringiensis* (Norris, 1964). A comparison of the biochemical, serological and esterase characteristics of crystal-forming bacteria.

Acetylmethyl-carbinol production	Lecithinase	Acid from salicin	Acid from sucrose	Hydrolysis of starch	H antigens	Serotype	Esterase pattern
+	+	+	+	+	I	1	Berliner
+	+	+	+	—	II	2	Finitimus
+	+	—	—	+	III	3	Alesti
+	+	—	+	+	IV (a)	4A	Sotto
+	+	—	—	+	IV (a)	4A	Dendrolimus
+	+	+	—	+	IV (b)	4B	Kenya
+	W*	+	—	+	V	5	Galleriae
—	—	—	+	+	VI	6	Entomocidus
+	—	—	+	+	VI	6	Entomocidus
+	+	+	—	+	VII	7	Galleriae
+	—	—	+	+	VIII	8	Morrison
+	+	+	—	+	IX	9	Tolworth

* W = reaction weak and variable.

(1965) have presented the case for separation. Consideration of additional evidence, i.e. mode of germination, components of the amino acid pool, surface morphology and biochemical reactions, would seem to justify its retention as a variety of *B. subtilis*. The sharing of a spore antigen with the latter (Table 1) is further evidence in support of the contention that it is a variant in which amylolytic and denitrifying enzymes are absent.

B. firmus and *B. lentus*

Knight and Proom (1950) have recorded difficulties in identifying new isolates of these glucose-sensitive species. Biochemically they show some striking affinities with organisms in group III. A more detailed study of a greater number of strains than hitherto examined may lead to a reappraisal of their status.

B. badius

In view of the very few strains examined we have omitted this organism from the scheme.

Group II (*Table* 5)

With one or two exceptions the species in this group are characterized by complex nitrogen requirements and by marked biochemical activity, e.g. caseolysis and fermentation. In this they resemble the members of group I and clearly contrast with those of group III. Another marked feature is the ability of most species to grow anaerobically, *B. brevis* being a noteworthy exception. The emphasis on the above features simplifies the problems raised by atypical strains of *B. polymyxa*, *B. macerans* and *B. pantothenticus*.

Reference has already been made to the frequent occurrence in this group of types intermediate between species. Some organisms may be so atypical as to be justifiably considered as equally disturbing misfits of as many as three different species. Such a tendency raises complications in the typing of strains. Further difficulties arise from the presence of inadequately defined species whose "present status is extremely unsatisfactory" (c.f. Smith *et al.*, 1952, on *B. circulans*), and from the presence of species which, on account of their inability to grow on commonly used media, largely depend on pathogenicity for their identification.

The primary subdivisions of the group separate (i) the two thermophilic species on the basis of temperature and pH, and (ii) *B. laterosporus* on its highly characteristic sporangial morphology.

B. stearothermophilus

A detailed study of over 200 cultures which included all the 75 strains of Smith, Gordon and Clark (1952) suggested the recognition of three distinct

subdivisions (Walker and Wolf, 1961, 1968). Their morphological and biochemical reactions are given in Table 10.

B. coagulans

Comments on this species appear under group I (see p. 100).

B. polymyxa

The sporangia are distinctly bulging, the spores are oval, terminal to subterminal and the spore wall is rather thin. Gas production and growth in an ammonium-glucose-salts medium are useful distinguishing characters. Biotin is an essential growth factor.

B. macerans

This organism differs from *B. polymyxa* in its additional requirement for aneurin. Gas production is more variable and erratic. Variants with properties intermediate between the *macerans-circulans-alvei* species are not infrequent.

B. larvae, *B. popilliae* and *B. lentimorbus*

The inability of these organisms to give adequate growth on common laboratory media is a poor criterion for identification. The use of such a negative characteristic is but a reflection of our ignorance of their more precise nutritional requirements. Recent interest in bacillary insect pathogens is beginning to rectify this sitation (e.g. Bailey and Lee, 1962).

B. brevis

The two outstanding characteristics of this species are the inability to grow anaerobically in glucose broth and the masking of glucose utilization by its potent proteolytic activity. This combination greatly facilitates identification.

B. pulvifaciens

The description of this species is based on the study of very few strains and an examination of a great many more isolates is clearly desirable.

B. pantothenticus

The strong reasons for accommodating this organism within this group have already been stated. Its dependence on pantothenic acid is highly characteristic.

B. circulans

The highly unsatisfactory status of this species has already been noted (Smith *et al.*, 1952).

TABLE 10. *B. stearothermophilus*. An examination of 230 cultures, which included all the seventy-five strains of Smith, Gordon and Clark (1952), indicated the existence of three distinct groups (Walker and Wolf, 1968)

Main biochemical reactions* of the three groups

	Group 1	Group 2	Group 3
Suggested identity	*B. kaustophilus* *B. thermodenitrificans*	New type or species	*B. stearothermophilus* *B. calidolactis* (lactose fermenters)
Morphology of spores	Oval to cylindrical	Oval	Oval to cylindrical
Swelling of sporangium	Slight to definite	Definite	Definite
3% NaCl broth	—	+	—
Hydrolysis of			
starch	RR	—	+ (Strong)
gelatin	+ or—	—	+ or—
casein	—	—	+ or—
NO_3—NO_2	+	—	+ or—
NO_2—gas	+	—	—
Tomato-yeast milk	Un:R or R & WC	Un	A.C.R.
Litmus milk	Un. or Sl. R	Un	Un. or A.C.R.
Growth in glucose anaerobically	Poor or negative	—	+
Acid from			
arabinose	+ or—	—	—
mannitol	+ or—	+	—
lactose	—	—	+ or—
xylose	+ or—	+	+ or—
rhamnose	+ or—	+	—
cellobiose	+ or—	+	+ or—

RR=restricted; Sl.=slight; A=acid; R=reduction; WC=weak curd; A.C.R. =acid, clot, reduction; Un=unchanged.

No growth on proteose-peptone acid agar; V.P.—; Indole—. All strains gave acid from glucose and maltose and most from sucrose.

*Incubation in a water bath at 55°.

B. alvei

Typical strains of this organism are readily identifiable on the basis of three morphological characters: (*a*) the palisade arrangement of vegetative cells and spores, (*b*) the presence of a distinctive exosporial membrane, easily seen with the light microscope when treated with a cell-wall stain, (*c*) the presence of satellite colonies around a point of inoculation.

Group III (*Table* 6)

The group, as now constituted, is characterized by the biochemical inertness of its two species and this has made it necessary to rely on a single physiological characteristic for their separation. Thus, *B. pasteurii* has an absolute requirement for both high pH and the presence of ammonia or urea.

B. pasteurii

There is evidence that the concentration of ammonia or urea required is strain specific and in the isolation of these organisms a gradation of concentrations must therefore be employed. The nitrogen requirements of this species are highly complex and require supplementation with growth factors as well as ammonia or urea, and as already observed by Knight and Proom (1950, p. 534), "The nutritional requirements of *B. pasteurii* suggest problems which require further study".

B. sphaericus

The species is distinguished from *B. pasteurii* by not requiring ammonia, urea or high pH. Its strictly aerobic nature deserves emphasis. Most strains grow readily at pH 6·0, but a minority, possibly representing distinct variants, require a higher pH (e.g. var. *rotans*). The presence of urease in some strains indicates further variants (e.g. var. *fusiformis* and var. *loehnisii*).

Media and Methods

With very few exceptions, the media used were those of Smith *et al.* (1952) and of Knight and Proom (1950). For convenience these are subsequently referred to as S.G.C. and K. & P., respectively.

Microscopy. Cultures grown on "Oxoid" nutrient agar slopes were used for most morphological observations. These were based on Gram stains, spore stains, and cell-wall stains (for the exosporium of *B. alvei*). Cultures on glucose agar were used for observations on vacuolation—as S.G.C.

Nutrient agar and nutrient broth. "Oxoid" brand (which contains some yeast extract) was used.

Retardation effects due to glucose. These were observed by comparing the extent of growth on nutrient agar slopes with and without glucose (1%).

pH. Growth was observed on appropriately adjusted "Oxoid" nutrient agar slopes or in nutrient broth.

Salt broth. "Oxoid" nutrient broth was used with varying concentrations of NaCl.

Hydrolysis of casein, starch and gelatin. Tests as S.G.C.

Lecithinase reaction. "Oxoid" egg yolk emulsion was used in nutrient agar at a final concentration of 5%.

J/

V.P., Indole and Urease. Tests as S.G.C.

Nitrate reduction. Tests as S.G.C.

Fermentation tests. Seitz filtered solutions of glucose and other carbohydrates were used at a final concentration of 0·5%, usually in nutrient broth. Where negative results were obtained cultures were retested in the same medium adjusted to pH 6·5. and with chloro-phenol-red (0·004%) as the indicator. Sugar broths for *B. coagulans*—as S.G.C.

Glucose-ammonium salts medium. As K. & P.

Anaerobic growth in glucose broth. As S.G.C.

Nutritional requirements. The ammonia-basal medium supplemented with amino acids or growth factors was used as K. & P.

Litmus milk and tomato-yeast milk. As S.G.C.

Alkaline media for B. pasteurii. Urea medium—as S.G.C.; ammonia media—see Gibson (1934 *b*).

Tests for thermophiles. Incubation was carried out in water baths at the specified temperatures.]

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Identification of Genera of the Actinomycetales

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Since 1950 over twenty new genera in the order Actinomycetales (the "actinomycetes") have been described. Descriptions of new genera appear frequently and it is difficult for workers who do not have a specialized interest in this group to become acquainted with these descriptions. The situation is further complicated by the lack of a single, generally accepted scheme of classification and many alternative ones are available. Considerable discussion has occurred on the validity of some of the more recently described genera and there are the inevitable nomenclatural problems. It is not intended here to discuss the many problems in the taxonomy of the Actinomycetales, but to attempt to provide non-specialists with information which could assist in the identification of the genera of this order. Some methods used in identification of actinomycetes are described and a list of all described genera (valid or otherwise), together with a simplified key, are given.

Methods

Morphological characteristics are widely used for the delimitation of genera of actinomycetes and some methods useful for the observation of these organisms are described below.

Cover-slip cultures (*Kawato and Shinobu*, 1959)

A sterilized cover slip is carefully inserted at an angle of about 45° into a suitable agar medium in a Petri dish, until about half of the cover slip is in the medium. An actinomycete is then inoculated along the line where the medium meets the upper surface of the cover slip. After a suitable incubation period, usually 10–14 days at 25° for mesophiles or 3–5 days at 50° for

thermophiles, the cover slip is carefully removed, its orientation in the medium being noted, and placed upwards on a slide. The organism grows on both the medium and in a line along the cover slip, the latter growth adhering to the cover slip when it is removed from the medium. Noting the orientation of the cover slip in the medium facilitates the distinction between "substrate mycelium" (that growing on the area of the cover slip covered by medium) and "aerial mycelium" (that growing on the area of the slip above the medium). This distinction is an important one in the identification of actinomycetes.

Slide cultures (*Colmer and McCoy*, 1950)

A suitable medium is pipetted while molten on to a sterile slide, two parallel strips of medium, about 2 mm apart, being made. The actinomycete is inoculated on to each strip and slides are incubated in damp sterile dishes for a suitable period. The organism grows in the medium, but also spreads across the slide in the space between the strips where observation of the growth can be made more easily.

Direct observation of unstained preparations

Both cover slip and slide cultures can be observed unstained. For some genera, especially those with fragile sporing structures (e.g. conidial chains of *Streptomyces*) observation of unstained preparations is essential, as important characteristics may be lost during fixing and staining procedures. Direct observation of colonies growing on media in plates can also provide useful information. A high-power ($\times 40$) objective lens with a long working distance is particularly useful for this purpose. Genera forming sporangia (e.g. *Actinoplanes*) may be observed in water growing on baits, such as pollen grains (Willoughby, 1966).

Fixation and staining procedures

Cover-slip cultures may be fixed and stained quickly and effectively using the following procedure:

(a) Fix the growth on the cover slip with a few drops of absolute methanol for 15 min.

(b) Wash with tap water and blot dry.

(c) Stain with crystal violet for 1 min.

(d) Wash and blot dry.

Before staining, slide cultures must first be dried by placing them over boiling water for about 5 min until the agar has dried. They can then be

stained with crystal violet as above. Sometimes Gram staining of actinomycetes can provide a clear distinction between spores and mycelium, as they may have different reactions.

Analysis of the cell-wall composition of actinomycetes

The recent work of Becker, Lechevalier and Lechevalier (1965) and Yamaguchi (1965) has shown that valuable taxonomic information can be provided by the analysis of the cell-wall composition of genera of actinomycetes. Data is available which, if considered together with morphological information, can lead to a more rational classification of actinomycetes. In certain instances it is also possible to use cell-wall analysis as a routine method for the identification of actinomycete genera. Becker, Lechevalier, Gordon and Lechevalier (1964) and Murray and Proctor (1965) were able to differentiate rapidly between strains of *Nocardia* and *Streptomyces*, which are sometimes difficult to separate on morphological criteria, by paper chromatography of whole-cell hydrolysates.

The Genera of the Order *Actinomycetales*

In Table 1 is given a complete list of described genera with their authors. The considerable increase in the number of genera in recent years is well illustrated here. For detailed information on the nomenclatural status of many of these generic names, the work of Lessel (1960) should be consulted.

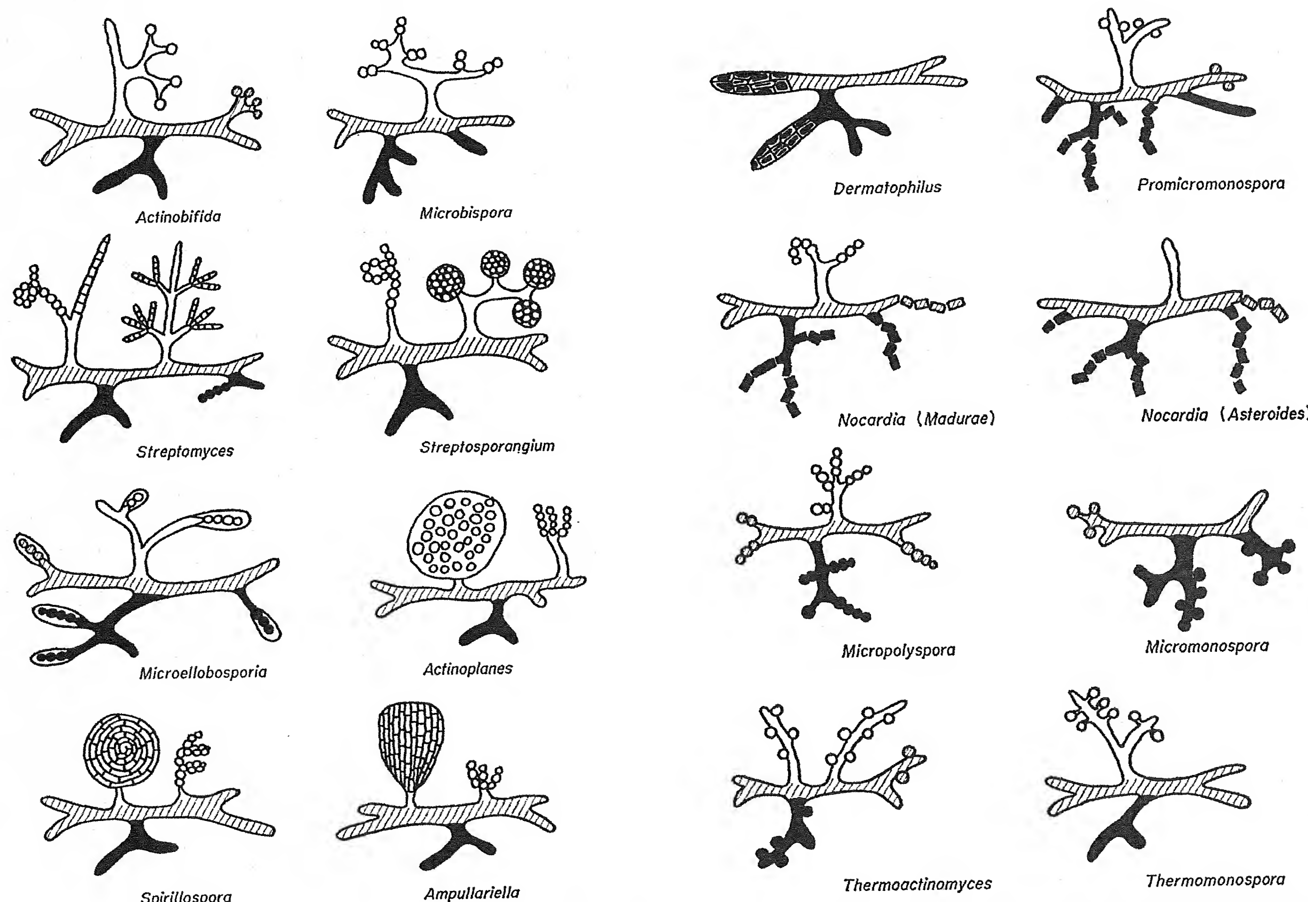
A Key for the Identification of Genera of the Actinomycetales

In the key given below, data on the cell-wall composition of the genera is taken from Lechevalier and Lechevalier (1965), unless otherwise indicated. These workers recognized four main types of cell-wall composition:

	Characteristic cell-wall components				
Cell-wall type	Glycine	LL-D.A.P.	Meso D.A.P.	Arabinose	Galactose
I	+	+			
II	+		+		
III			+		
IV			+	+	+

D.A.P. — $\alpha\epsilon$ diaminopimelic acid.

Cell-wall compositions marked * below are from Yamaguchi (1965).



FIGS 1 and 2. Schematic illustrations of actinomycete genera. The white portion represents the aerial mycelium, the cross-hatching identifies the substrate mycelium which is on the surface of the medium, and the dark filaments and spores represent the parts of the culture located in the agar. (After Lechevalier, 1964.)

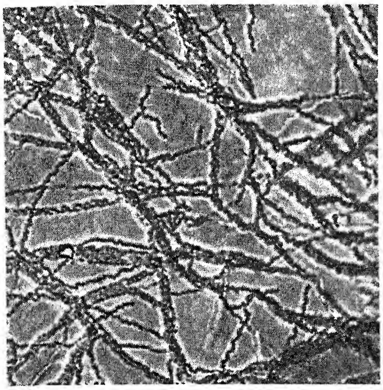


FIG. 3

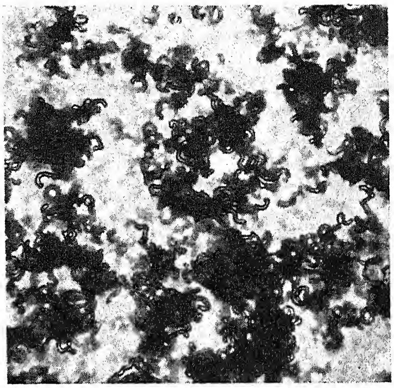


FIG. 4

FIG. 5

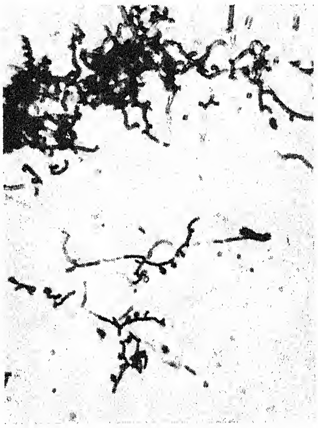


FIG. 6

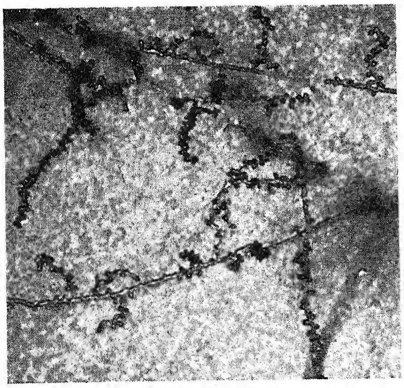


FIG. 7

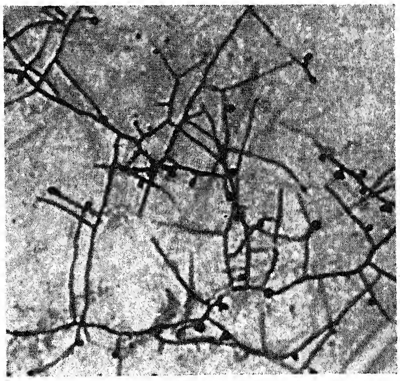


FIG. 8

FIGS 3 to 19, Photographic illustrations of some actinomycete genera.

FIG. 3. *Nocardia* type *madurae*, substrate mycelium (× 250).

FIG. 4. *Nocardia* type *madurae*, aerial mycelium (× 400).

FIG. 5. *Nocardia* type *asteroides*, substrate mycelium (× 500).

FIG. 6. *Thermoactinomyces vulgaris*, spores on aerial mycelium (× 500).

FIG. 7. *Thermomonospora viridis*, spores on aerial mycelium (× 400).

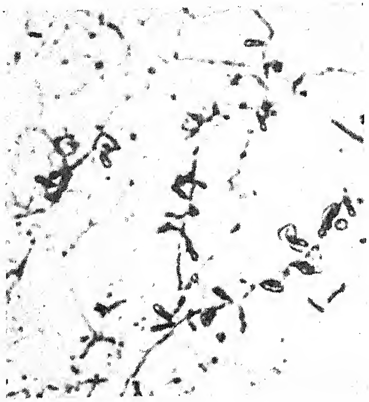


FIG. 9

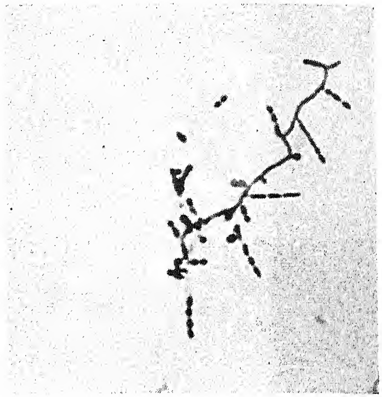


FIG. 10

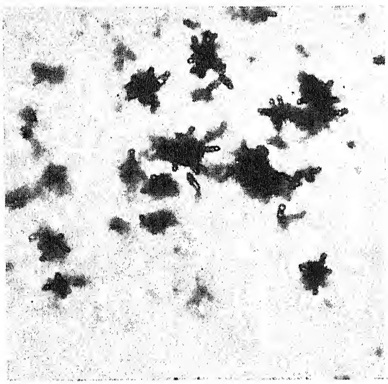


FIG. 11

FIG. 12

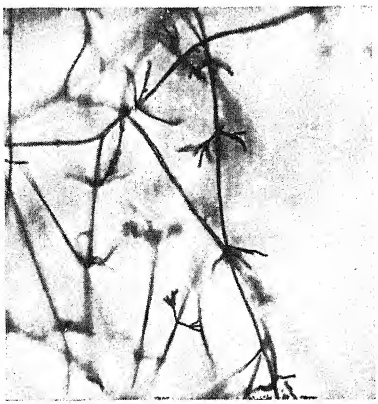


FIG. 13

FIG. 14

FIG. 9. *Microbispora rosea*, spores on aerial mycelium (× 1000).
FIG. 10. *Micropolyspora* sp., spores on substrate mycelium (× 1000).
FIG. 11. *Micropolyspora* sp., spores on aerial mycelium (× 400).
FIG. 12. *Streptomyces* sp., spiral spore chain on aerial mycelium (× 450).
FIG. 13. *Streptomyces* sp. (*Streptoverticillium* sp.), spore chains in verticils on aerial mycelium (× 250).

FIG. 15

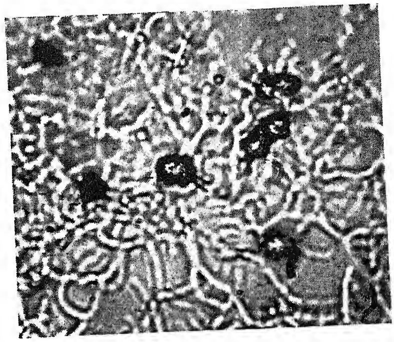


FIG. 16

FIG. 17

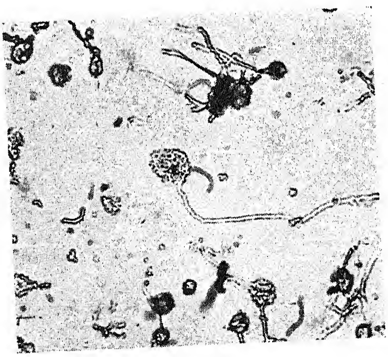


FIG. 18

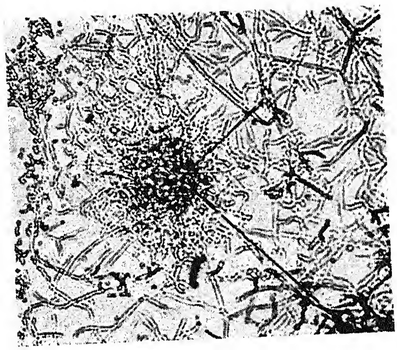


FIG. 19

FIG. 15. *Actinopycnidium* sp. (*Streptomyces* sp.), "pycnidia" and spore chains (× 375).
FIG. 16. *Actinoplanes* sp., sporangia on substrate mycelium (× 500).
FIG. 17. *Microellobosporia cinerea*, sporangia on aerial mycelium (× 1000).
FIG. 18. *Streptosporangium* sp., sporangia on aerial mycelium (× 500).
FIG. 19. *Actinosporangium* sp. (*Streptomyces* sp.), "sporangium" (× 225).

TABLE 1. List of all described genera of the order *Actinomycetales* (October 1966)

Actinomyces	Harz (1877)
Nocardia	Trevisan (1889)
Mycobacterium	Lehmann and Neumann (1896)
Thermoactinomyces	Tsiklinsky (1899)
Micromonospora	Ørskov (1923)
Proactinomyces	Jensen (1931)
Mycococcus	Krassil'nikov (1938)
Streptomyces	Waksman and Henrici (1943)
Actinoplanes	Couch (1950)
Jensenia	Bisset and Moore (1950)
Chainia	Thirumalachar (1955)
Streptosporangium	Couch (1955)
Microbispora	Nonomura and Ohara (1957)
Waksmania	Lechevalier and Lechevalier (1957)
Pseudonocardia	Henssen (1957)
Thermomonospora	Henssen (1957)
Thermopolyspora	Henssen (1957)
Actinomonospora	Castellani, De Brito and Pinto (1958)
Dermatophilus	(van Saceghem) Austwick (1958)
Streptoverticillium	Baldacci (1959)
Actinosporangium	Krassil'nikov and Tsi Shen (1961)
Micropolyspora	Lechevalier, Solotorovsky and McDurmont (1961)
Promicromonospora	Krassil'nikov, Kalakoutski and Kirillova (1961)
Actinopycnidium	Krassil'nikov (1962)
Thermoactinopolyspora	Craveri and Pagani (1962)
Amorphosporangium	Couch (1963)
Ampullariella	Couch (1963) (1964)
Macrospora	Tsyganov, Jukova and Timofieva (1963)
Microellobosporia	Cross, Lechevalier and Lechevalier (1963)
Spirillospora	Couch (1963)
Actinobifida	Krassil'nikov and Agre (1964)
Microechinospora	Konev, Tsyganov, Minbaev and Morosov (1965)
Odontomyces	Howell, Jordan, Georg and Pine (1965)
Elytrosporangium	Falcão de Morais, Cheaves Batista and Massa
Waksmara	No valid publication

I. Mycelium rudimentary or absent. Branched cells may or may not be formed. True mycelium not formed on the surface of organic media, but occasionally found in clinical material. Strict aerobes. Spores never formed.

A. Cells usually acid fast. Rod-shaped cells that do not branch under normal laboratory conditions. *Mycobacterium*.

B. Cells non-acid fast. Cells tend to be spherical, occurring singly or in clumps. *Mycococcus*.

II. Substrate mycelium transitory and fragmenting into bacillary or coccoid elements. Aerobic to anaerobic.

A. No aerial mycelium. True mycelium formed on the surface of the agar media and may be transitory. Anaerobic to facultative, catalase negative. Characteristic components of cell wall are lysine, glutamic acid, alanine and galactose.* *Actinomyces*.

B. No aerial mycelium. True mycelium formed on the surface of agar media, transitory. Facultative to anaerobic, catalase positive. *Odontomyces*.

C. No aerial mycelium. True primary mycelium formed which divides in all planes to give coccoid elements. Motile spores may be formed by longitudinal and transverse division of hyphae. Aerobic to facultative. Cell-wall type III. *Dermatophilus*.

D. No or very little aerial mycelium. Single spores on short sporophores or sessile on branches of substrate mycelium. Characteristic components of cell wall are lysine, glutamic acid, alanine and galactose.* *Promicromonospora*.

E. Aerial mycelium present or absent. When present, chains of spherical conidia produced. Aerobic. Cell-wall type III. *Nocardia* type *Madurae*.

F. Aerial mycelium rarely visible. Conidia absent or if present, poorly developed and difficult to see. Marked fragmentation of substrate mycelium. Aerobic. Cell-wall type IV. *Nocardia* type *Asteroides*.

G. Aerial mycelium present. Substrate mycelium on agar not normally fragmenting. Fragmentation may occur when preparing smears. Conidia formed in short lateral and terminal chains (occasionally singly) on both substrate and aerial mycelium. Aerobic. Cell-wall type IV. *Micropolyspora*.

III. Substrate mycelium stable, not fragmenting. Aerobic.

A. Spores are conidia, not formed in sporangium.

(i) Single conidia formed.

(*a*) No aerial mycelium. Single conidia sessile or on the tip of conidiophores which may branch. Cell-wall type II. *Micromonospora*.

(*b*) Aerial mycelium formed. Conidia on short unbranched conidiophores usually formed on aerial mycelium only. Cell-wall type IV. *Thermomonospora*.

(*c*) Aerial mycelium formed. Conidia usually appearing sessile on both aerial and substrate mycelium. Cell-wall type III. *Thermoactinomyces.*

(ii) Paired conidia formed on aerial mycelium only. Cell-wall type III. *Microbispora.*

(iii) Conidia in chains on the aerial mycelium and occasionally on substrate mycelium. Cell-wall type I. *Streptomyces.*

B. Spores formed in sporangia (5–30 μm diameter), chains of conidia may also be present.

(i) Spores motile with flagella.

(*a*) No aerial mycelium, sporangia spherical to irregular, spores sub-spherical. Chains of conidia may also be present. Cell-wall type II. *Actinoplanes.*

(*b*) No aerial mycelium, sporangia cylindrical to bottle-shaped, spores rod-shaped. Brush-like conidiophores may be present. Characteristic cell-wall components meso-and/or DD-D.A.P. and smaller amounts of LL-D.A.P.; glutamic acid and alanine, pentoses present or not.* *Ampulariella.*

(*c*) Aerial mycelium present, sporangia spherical, spores rod- to spiral-shaped. Coils of conidia may be present. Cell-wall type II. *Spirillospora.*

(ii) Spores non-motile.

(*a*) Aerial mycelium present, sporangia spherical, spores sub-spherical to rod-shaped. Chains of conidia also formed. Cell-wall type II. *Streptosporangium.*

(*b*) Aerial mycelium present, sporangia very irregular, spores rod-shaped. Cell-wall type II. *Amorphosporangium.*

(*c*) Aerial mycelium present, sporangia club-shaped (2–9 μm long, 1·5–3·6 μm diameter), containing a single row of spherical spores. Sporangia formed on substrate and aerial mycelium. Cell-wall type I. *Microellobosporia.*

Of the genera given in this key, two, *Thermomonospora* and *Thermoactinomyces*, are entirely thermophilic, e.g. *Thermomonospora viridis* strains have an optimum temperature for growth in the range of 45–50°, *Thermoactinomyces vulgaris* strains have an optimum temperature in the range

50–60°, but will produce visible colonies on certain media if incubated at 37° or even 30°. However, thermophily also occurs in some species of other genera, including *Nocardia*, *Streptomyces* and *Micropolyspora*.

Certain genera are excluded from the key because insufficient information is available to assess their validity. These are as follows:

Actinobifida. Members of this genus are reported to produce a well-defined aerial mycelium bearing dichotomously branched conidiophores terminating in single spores. Species of this genus could possibly be included in the genus *Thermomonospora*.

Microechinospora. A single species of this genus has been reported which bears spiny sporangia on the aerial and substrate mycelium which contain a single spore or occasionally a short chain of spores. It seems to be close to *Microellobosporia*.

Pseudonocardia. Aerial mycelium is formed with long conidia (2·5 μm) in chains. Spores also form on the septate fragmenting substrate mycelium. Cell-wall type IV. This is possibly a thermophilic *Nocardia*.

In Table 2 is given a list of genera excluded from the key, together with reasons for their exclusion.

TABLE 2. List of genera excluded from key

Jensenia	Forms of *Corynebacterium*, *Mycobacterium*, and *Nocardia*
Chainia	*Streptomyces* forming sclerotia-like structures Cell-wall composition as *Streptomyces*
Streptoverticillium	*Streptomyces* with conidiophores in verticils Cell-wall composition as *Streptomyces*
Actinopycnidium	Streptomyces forming pycnidia-like structures Cell-wall composition as *Streptomyces*
Actinosporangium	Streptomyces forming "pseudo-sporangia". Cell-wall composition as *Streptomyces*
Thermopolyspora	Thermophilic *Microbispora*, *Streptomyces* and *Nocardia*
Thermoactinopolyspora	Thermophilic *Streptomyces*
Waksmania	Synonym of *Microbispora* (Lechevalier, 1965)
Macrospora	Synonym of *Microellobosporia*
Actinomonospora	Synonym of *Micromonospora*
Proactinomyces	Synonym of *Nocardia* or *Actinomyces*
Waksmara	Aquatic forms of *Streptomyces*. Name not validly published, but described by Roach and Silvey (1958)

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Note

Several new genera have been described since this paper was completed in October, 1966. Details of these genera are given below without critical appraisal of their status. Some additional information pertaining to the genera listed above is also included.

Actinobifida. Three strains of the species *Actinobifida dichotomica* have been found to bear clusters of single spores on the substrate mycelium in addition to the spores on the aerial mycelium. Baldacci, E. and Locci, R. (1966). *Giorn. Microbiol.*, **14,** 131. Cross, T. (1968). *J. appl. Bact.*, **31,** (in press).

Elytrosporangium. Morais, J. O. Falcao de, Cheaves Batista, A. and Massa, D.M.G. (1966). *Mycopath. Mycol. Appl.*, **30,** 161.

This organism produces *Streptomyces-like* spore chains on the aerial hyphae and pod-shaped sporangia, containing non-motile spores on the substrate hyphae. Cell wall type I. See also Morais, J. O. Falcae de (1967). *Hindustan Antibiot. Bull.*, **9,** 135.

Dactylosporangium. Thiemann, J. E., Pagani, H. and Beretta, G. (1967). *Arch. Mikrobiol.*, **58,** 42.

This organism produces finger-shaped sporangia emerging directly from the substrate mycelium. The sporangia contain a single straight row of motile spores. No aerial mycelium is formed. The authors also mention the isolation of two further genera, *Planomonospora* with fusiform sporangia on aerial mycelium containing a single rod-shaped zoospore and *Microtetrospora*, with aerial mycelium bearing short chains of four spores.

Intrasporangium. Kalakoutski, L. V., Kirillova, I. P. and Krassilnikov, N. A. (1967). *J. gen. Microbiol.*, **48,** 79.

Intercalary sporangia are formed on the substrate mycelium and contain non-motile spores. No aerial mycelium is formed and colonies are round and glistening, resembling bacteria.

Pilimelia. Kane, W. D. (1966). *J. Elisha Mitchell scient. Soc.*, **82,** 220.

Sporangia are formed on the aerial mycelium and contain motile, rod-shaped spores with a single polar flagellum. The spores are arranged in parallel chains within the sporangium.

Rothia. Georg, L. K. and Brown, J. M. (1967). *Int. J. Syst. Bact.*, **17,** 79.

Mycelia fragment rapidly into bacillary or coccoid elements. No aerial mycelium or spores are formed and all elements are non-motile. Growth is good under aerobic or microaerophilic conditions but poor in anaerobic conditions. Catalase positive.

Methods for the Identification of Mycoplasmas

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The mycoplasmas constitute a group of micro-organisms which are distinguished from bacteria by the lack of a cell wall, the size of the smallest reproductive forms (125–150 mμ or strictly nm), and a characteristic colony form. With the exception of saprophytic strains belonging to one species, mycoplasmas require serum or ascitic fluid for growth, the essential requirements being protein, sterol and phospholipid.

At present, they are classified in a single genus (*Mycoplasma*), family (Mycoplasmataceae), and order (Mycoplasmatales) in the newly proposed class Mollicutales. Only fifteen species are listed in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1957), but the tremendous upsurge of interest in this group in recent years has resulted in the isolation of a large number of new species, not all of which have yet been named. A list which includes most of the recently named species is given in Table 1. The nomenclature of others is still under discussion. Moreover, as species become better characterized, it may be necessary to recognize more than one genus.

Nevertheless, in spite of current taxonomic uncertainties, in practice it is possible to distinguish individual species of *Mycoplasma*. For many species, representative strains are available with which new isolates can be compared (Edward and Freundt, 1956) and with the setting up of national and international reference laboratories, authentic cultures of these and more recently recognized species should be readily obtainable.

At present, serological techniques are the most reliable for identifying members of the genus, with biological characters providing useful supporting data. DNA homology tests for determining the genetic relationships

*Present address: Mycoplasma Reference Laboratory, Central Public Health Laboratory, London, N.W.9, England.

TABLE 1. Named species of *Mycoplasma*

Species	Main Source
M. pneumoniae	Man
M. hominis	
M. salivarium	
M. pharyngis } synonyms	
M. orale } synonyms	
M. orale, type 2*	
M. fermentans	
M. mycoides var. *mycoides*	Cattle†
M. bovigenitalium	
M. bovirhinis	
M. mycoides var. *capri*	Goats or sheep
M. agalactiae	
M. hyorhinis	Pigs
M. granularum	
M. hyopneumoniae } synonyms	
M. suipneumoniae } synonyms	
M. spumans	Dogs
M. maculosum	
M. canis	
M. pulmonis	Rats or mice
M. arthritidis	
M. neurolyticum	
M. gallisepticum	Poultry†
M. gallinarum	
M. synoviae	
M. meleagridis	
M. iners	
M. anatis	
M. laidlawii	Soil, sewage, some animals (presumed saprophyte)

*Tentative name.

†From these sources several other distinct serological types have been characterized, but not yet named.

of different organisms within the genus have been successfully developed recently (Rogul *et al.*, 1965); Reich *et al.*, 1966). However, as these techniques are highly specialized and not suitable for routine use, they are not dealt with here. The results of DNA homology tests have so far confirmed those obtained by serological typing.

Distinguishing *Mycoplasma* Colonies from those of Bacteria

The mycoplasmas have a characteristic colony form which distinguishes them from bacteria. This normally consists of a central zone embedded in the agar and a peripheral zone, representing secondary growth over the surface (Razin and Oliver, 1961, Edward, 1954). This so-called "fried egg" form is most characteristically seen if the colony is viewed at low magnification, for example with a dissecting microscope using oblique transmittted light (Edward, 1954). Scraping off the surface growth with a loop leaves the buried centre intact. Despite the basic colony form, there is some variation in colonial appearance as between different species of *Mycoplasma*. However, colonial morphology cannot be relied upon as a criterion for differentiation within the genus, because it varies with the constitution of the medium, the firmness of the agar gel and the amount of moisture present.

When viewed at high magnification, in preparations fixed through agar with Bouin's solution and stained with Giemsa (Klieneberger-Nobel, 1962), young colonies are seen to consist of delicate, apparently flattened cells, which are variable in shape, size and staining intensity. Some are filled with densely staining material; in others the stainable material is aggregated into discrete granules.

Dienes's method of examining colonies *in situ* under a stained cover slip (Madoff, 1960) is more rapid, but shows less structure than the method of Klieneberger-Nobel. It is, however, very useful for detecting small mycoplasma colonies (i.e. those in an early stage of development) and those of the so-called "T-strain mycoplasmas" (T=tiny).

Distinguishing *Mycoplasma* Colonies from those of Bacterial L-forms

The isolation of mycoplasmas is frequently attempted on media containing antibiotics and other bacterial inhibitors. Such conditions may cause some bacteria to lose all or part of their cell wall and to give rise to L-form colonies which resemble those of mycoplasmas.

Mycoplasmas can be distinguished from L-forms by the stability of their colony form when subcultured on media without bacterial inhibitors. When L-forms derived from bacteria are transferred to media without inhibitors they usually give rise to colonies of the parent bacterium, providing these transfers are carried out immediately. If subculture in the absence of inhibitors is left to a later stage after isolation, an L-form may become incapable of reversion to the bacterial form.

Macroscopically, L-form colonies are usually coarser and more opaque than those of mycoplasmas, and these differences become more pronounced

if incubation of the cultures is prolonged. In liquid media, growth of L-forms is frequently granular, with a density approaching that of the parent bacterium. This is in contrast to fluid cultures of mycoplasmas which become only slightly turbid.

The microscopic appearance of Bouin–Giemsa preparations can also indicate to an experienced worker whether he is dealing with a mycoplasma or an L-form. Mycoplasma cells are usually more delicate and stain less intensely than those in L-form colonies. The latter also have a more amorphous appearance (Klieneberger-Nobel, 1962).

Serological Methods for Identification within the genus *Mycoplasma*

Sera are first prepared against representative strains of appropriate species. In practice, as mycoplasmas are largely host-specific, the source of isolation determines the range of antisera against which the unknown mycoplasma is tested.

Various systems for growing the antigen and for immunizing rabbits have been devised (Butler and Leach, 1964; Lemcke, 1964; Taylor-Robinson *et al.*, 1963). It is important to avoid production of antibody to components of the medium. In particular, rabbit serum should be used in the medium when growing organisms intended for immunization of rabbits.

Many of the serological techniques used in other branches of microbiology are also applicable to the mycoplasmas. Methods which may be of use in identification are described below.

Growth-inhibition by specific antisera

The growth of mycoplasmas is inhibited by specific antisera. This phenomenon forms the basis of two highly specific methods for identifying mycoplasmas.

Inhibition of growth on agar

The test originally devised by Edward and Fitzgerald (1954) has been modified by Huijsmans-Evers and Ruys (1956) and Clyde (1964). Antisera to known species are absorbed into filter-paper discs which are then superimposed on plates of suitable medium which have been inoculated with dilutions of a culture of the unknown mycoplasma designed to produce a dense, but not confluent, growth of colonies after an appropriate incubation period (2–4 days). Zones of growth-inhibition are produced only around discs containing antiserum to the same species as the unknown.

When antisera of high potency are available, this is the simplest and most convenient of the serological methods for identification of mycoplasmas; it is also one of the most specific.

Metabolic inhibition tests

These tests are dependent on the fact that the majority of mycoplasmas are able either to ferment glucose and certain other carbohydrates or to metabolize arginine to ammonia. One group, the "T-strain mycoplasmas", metabolizes urea to ammonia. These reactions cause pH changes, and therefore colour changes, in media containing the appropriate substrate and phenol red. Specific antisera inhibit these colour changes. In metabolic inhibition tests, a mixture of mycoplasma culture and the appropriate medium is incubated with a range of dilutions of antiserum (Taylor-Robinson *et al.*, 1966; Purcell *et al.*, 1966*a*, *b*). The colour change produced by normal growth of the mycoplasma is inhibited only by antisera to strains of the same species. Some species are also able to reduce tetrazolium salts to coloured formazan compounds, a reaction which can be used as the basis of another metabolic inhibition test (Jensen, 1964).

Agglutination

Mycoplasma suspensions are directly agglutinable by specific antiserum. Agglutination may be demonstrated by tube tests or by slide or plate tests. Like the growth inhibition test, it distinguishes clearly between species of *Mycoplasma* and tends to be more specific than most other serological methods including complement fixation. Strains showing a relationship by complement fixation, e.g. different strains of *M. hominis*, isolated from the human genital tract (Nicol and Edward, 1953; Edward, 1954), may fail to cross react by agglutination.

Mycoplasma suspensions for agglutination tests are usually prepared from fluid cultures by centrifugal concentration and resuspension in saline (0·85% w/v), after which they are standardized at an opacity approximately that of Brown's Tube Nos 1 or 2. With some strains, gentle mechanical homogenization may help towards obtaining a suitable suspension. Preservatives such as formalin or phenol may be added. The method recommended by Edward (1954) for preparation of antigens, *viz.* use of fluid medium as a thin overlay on solid medium, often provides particularly suitable suspensions. Tests can be carried out in the conventional way against diluted sera in Dreyer tubes and read, by the naked eye or with a hand lens, after 4–6 h at 52° or overnight at 37°.

Agglutination can be performed as a rapid spot test on a glass slide or plate, using as antigen a more concentrated mycoplasma suspension which

may be stained with a suitable dye such as Crystal Violet or Rose Bengal. This type of test is used mainly for serological diagnosis of mycoplasma infections, particularly in poultry (Adler, 1958).

Complement fixation test

This method is somewhat less specific than the growth-inhibition and agglutination tests and cross-reactions may occur at low titre between strains of different species. Nevertheless, with high-titre antisera, prepared with due regard to avoiding antibody to constituents of the medium, unrelated types are clearly distinguishable.

Antisera which are inadequate for identification of a mycoplasma by agar growth-inhibition and agglutination tests often give satisfactory results in complement fixation tests.

Mycoplasma antigens for complement fixation tests usually consist of suspensions of whole cells in saline (0·85%, w/v), or veronal-buffered saline (e.g. Barbitone Complement Fixation Test Diluent prepared from tablets (Oxoid)), preserved with merthiolate (Lilly's Solution No. 45, final concentration 1 in 10,000) or sodium azide (0·08% w/v). With certain strains possessing heat-stable antigens (e.g. *M. mycoides* and *M. pneumoniae*) heat-treated suspensions may prove superior, but it is advisable at first to carry out tests with unheated suspensions of an unknown mycoplasma.

To conserve serum and antigen, micro-serological systems such as those manufactured by the Cooke Engineering Co., Alexandria, Va., U.S.A. (Microtiter) and Shandon Scientific Co. Ltd, London, England (Microtitrator) can be used.

Fluorescent antibody technique

Colonies of mycoplasma growing on solid media can be identified by this method, but as it has no advantage over simpler techniques, it is probably of greater value for identification of mycoplasmas in tissues or tissue cultures.

Colonies on agar

Agar blocks are placed on slides with the colony-bearing surface downward. The slide is immersed in water at 85° and, as the agar begins to melt, the block can be shaken off, leaving the colonies fixed on the slide (Clark *et al.*, 1961). After air-drying, the colonies are fixed in dry acetone for 10 min (Chanock *et al.*, 1962). The indirect method is usually employed, the rabbit antiserum being applied first, then the fluorescein-conjugated anti-rabbit globulin. It is advantageous to adsorb both antiserum and conjugated globulin with acetone-dried horse-liver powder and washed yeast cells to eliminate non-specific fluorescence due to medium constituents (Lemcke *et al.*, 1965). It is also important to use young colonies; the fluorescence of old

colonies with homologous antisera is less intense and occurs only at the edges of the colonies.

Mycoplasmas in tissue or tissue cultures

Both the direct and indirect methods are used. Tissues are best examined as cold microtome sections, obtained from frozen material (Liu, 1957; Goodburn and Marmion, 1962). Tissue cultures are most conveniently studied as monolayers grown on cover slips (Malizia *et al.*, 1966). Preparations are usually air-dried and fixed in dry acetone. The method can be used also for the identification of mycoplasmas in clinical material (Noel *et al.*, 1964). It is probably more suitable as a means of identifying strains known to be present in tissues, but difficult to isolate, than as a screening method for the presence of mycoplasmas.

Other serological tests

Agar gel diffusion

Because of difficulties in preparing suspensions which react consistently and the necessity for potent antisera, this technique has disadvantages for routine identification. Nevertheless, it is useful for demonstrating small antigenic differences between strains of the same species. Suitable antisera can be prepared with Freund's adjuvant. Antigens consist of concentrated mycoplasma suspensions disrupted by physical methods (Lemcke, 1965*a*; Taylor-Robinson *et al.*, 1963, 1965).

Other serological techniques applicable to mycoplasmas include haemagglutination-inhibition (Feldman and Suhs, 1966; Yoder and Hofstadt, 1964) and indirect haemagglutination (Cottew, 1960; Dowdle and Robinson, 1964), but these are not normally used for primary identification.

Choice of serological techniques

The choice of techniques must rest ultimately on the individual worker and the facilities available, but it is usually preferable to employ at least two methods in identifying a species. For routine identification the growth-inhibition test on agar is the simplest and most convenient. If sera with high growth-inhibitory powers are available, this test alone may suffice, but it is usually advisable to confirm the identity of the organism by another method. Agglutination, which has the advantage of simplicity, can be used for confirmatory tests, but in view of the excessive specificity of that method (see above) the authors prefer the complement fixation technique for this purpose.

Biological Properties

Although the metabolic activities of certain mycoplasmas have been intensively studied (Smith, 1964), there is a paucity of detailed information

about most species. This may account for the fact that the number of biological properties used to characterize mycoplasmas is relatively small. The necessity for adding to the number of available tests and standardizing the conditions under which biochemical characteristics are determined was recently emphasized by Fabricant and Freundt (1967), who pointed out that the end result of certain tests depended on the constitution of the medium.

At present, therefore, a mycoplasma cannot be identified solely on the basis of its biological characters; identification must rest ultimately on the results of serological tests. However, biological characters are useful for characterizing a strain and giving some indication of its possible identity.

There is no publication which lists the biological properties of all the recognized species of *Mycoplasma*. For details regarding particular species it is therefore necessary to refer to the literature pertaining to the appropriate group, e.g. mycoplasmas from man (Hayflick and Chanock, 1965); from poultry (Yoder and Hofstadt, 1964) and from other sources (Edward, 1954; Freundt, 1958).

The following tests are frequently used in the determination of biological properties.

Ability to grow without serum

Some mycoplasmas are able to grow in artificial media in the absence of added serum components. These strains, which are also characterized by an ability to grow at low temperature (22°), belong to a single serological group, the species *M. laidlawii*. These strains are generally regarded as saprophytic (Edward, 1954), as they are found in sewage and soil. However, as they have sometimes been found in animal hosts, their serum and temperature requirements provide useful screening tests in distinguishing them from parasitic strains.

Carbohydrate fermentation

Mycoplasmas can be divided into fermentative and non-fermentative strains. For this purpose it is generally sufficient to use glucose alone; the range of other carbohydrates utilized is similar for all the fermentative strains and cannot be used for further differentiation (Tourtellotte and Jacobs, 1960). Glucose fermentation may be tested in either solid, semi-solid or fluid media. A useful medium consists of Difco PPLO broth; fresh yeast extract (2·5% w/v); phenol red (0·002% w/v); glucose (1·0% w/v); horse serum (20% w/v). When the initial pH is approximately 7·8, acidification due to fermentation is indicated by a change in colour from pink to orange or yellow. In addition to uninoculated test medium, controls should include cultures in the same medium but without added glucose. This is

important, since some strains may cause pH changes in the medium even in the absence of added sugar (Freundt, 1958).

Potentially useful supplementary tests are provided by the recent demonstration that non-fermentative species of *Mycoplasma* may utilize either arginine or, as with the "T-strains," urea (Purcell *et al.*, 1966*a*, *b*). When either of these two metabolites is substituted for glucose in the above formula, and the initial pH of the medium adjusted to 7·2 for arginine and 6·0 for urea, growth of the mycoplasmas is accompanied by an alkaline shift and consequent change in colour from yellow or orange to pink.

The formation of a "film and spots"

When the incubation period is prolonged sufficiently, certain species of *Mycoplasma* produce an irridescent ("pearly") film, together with dark spots in the depths of solid medium containing 20% horse serum (Edward, 1954; Freundt, 1958). This is associated with the degradation of lipids in the medium. Edward obtained consistent results with ox-heart infusion-peptone agar medium containing 20% inactivated horse serum and 1% (w/v) Oxoid yeast extract, the cultures being incubated for at least 6 days at 37°.

Haemolysis

Some mycoplasmas produce changes in blood agar, poured as an overlay on discrete colonies already growing in agar. These changes, which occur aerobically, may take the form of partial haemolysis producing faint plaques in the overlay, or complete haemolysis with distinct zones of clearing which resemble β-haemolysis. Guinea-pig and sheep erythrocytes give the best results and should be applied as a 4% blood agar overlay to discrete colonies which have already developed (Clyde, 1963; Somerson *et al.*, 1963). Haemolysis develops after further incubation for 18–48 h aerobically at 37°.

Haemadsorption

Although tissue cultures infected with certain mycoplasmas may be able to adsorb erythrocytes (Berg and Frothingham, 1961), *M. pneumoniae* is the only species so far recorded as capable of adsorbing erythrocytes directly on to its colonies (Del Guidice and Pavia, 1964). This property distinguishes it from other mycoplasmas of human origin. Colonies on plates flooded with a 1% suspension of washed sheep erythrocytes and incubated for 30 min at 37° retain a coating of erythrocytes when the suspension is washed off with saline.

Tetrazolium reduction test

Several species are known to reduce 2,3,5-triphenyl-tetrazolium chloride anaerobically (Somerson and Morton, 1953), but *M. pneumoniae* can be

distinguished from other mycoplasmas of human origin by its ability to reduce tetrazolium aerobically to a pink formazan compound in solid or liquid media (Kraybill and Crawford, 1965). The medium described for carbohydrate fermentation (above) is used with the test substance, at a final concentration of 0·02% (w/v), substituted for glucose and the phenol red omitted. The liquid medium is inoculated with 10% of its volume of an actively growing culture. Solid medium is inoculated with agar blocks bearing a heavy growth of mycoplasma colonies; when tetrazolium is reduced, a pink colour develops under the agar block.

Appendix

There are a number of media for growing mycoplasmas (Lemcke, 1965*b*). The following formula is widely used: Difco PPLO agar containing fresh yeast extract (2·5% w/v) and unheated horse serum (20% w/v); final pH 7·8. Penicillin (100 units/ml) and thallous acetate (1/8000) may be included to inhibit the growth of contaminating bacteria. The corresponding liquid medium is prepared with Difco PPLO broth (without Crystal Violet).

These basic media can be supplemented with appropriate substrates such as glucose, arginine, urea or 2,3,5-triphenyl-tetrazolium chloride for biochemical tests.

Mycoplasma cultures are normally incubated at approximately 37° and should be grown anaerobically as well as aerobically when first examined, so that optimal conditions can be used in subsequent identification tests. Incubation in an atmosphere of 5–10% CO_2 in nitrogen is often used as an alternative to conventional anaerobiosis in hydrogen. Cultures on solid medium should be incubated in a moist atmosphere in order to avoid drying of the medium.

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Note

Since this work was completed in October 1966, several useful reports have been published.

Fluorescent antibody technique

Del Guidice, R. A.,Robillard, N. F. and Carski, T. R. (1967). *J. Bact.*, **93,** 1205.

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The first of these describes the recognition of mixed cultures of mycoplasmas on agar, the colonies being examined *in situ* after treatment with fluorescein-conjugated globulin from specific antisera.

Preparation of antisera to *Mycoplasma* species

A new method has been described and standard methods evaluated by Morton, H. E. and Roberts, R. J. (1967). *Proc. Soc. exp. Biol. Med.*, **125,** 538.

Haemadsorption

This has now been reported to occur in species other than *M. pneumoniae* by Taylor-Robinson, D. and Manchee, R. J. (1967). *Nature, Lond.*, **215,** 484.

The culture of the Blue-Green Algae and the Use of the Electron Microscope in Identification and Classification

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The blue-green algae differ from other algae in that they consist of procaryotic cells, a feature which they share with the bacteria. Such organisms lack the organized nucleus and well-known divisional mechanisms of higher (eucaryotic) cells, and do not have the internal membranous compartments of mitochondria, plastids, dictyosomes and endoplasmic reticulum. The genetic material in the procaryotic cell consists of a finely fibrillar nucleoplasm which is centrally located, and the various metabolic functions normally ascribed to the recognizable organelles in the cytoplasm of higher organisms are located on or near the membranes within the procaryotic cell. Although the procaryotic cells lack the structural complexity of the eucaryotes, they do nevertheless contain the sophisticated biochemical machinery of the higher forms.

The blue-green algae are similar in many respects to the bacteria, and in particular to the unicellular forms. The relationship between the two groups is not clearly understood and Pringsheim (1949) and, more recently, Echlin and Morris (1965) have summarized the morphological and biochemical similarities and differences between these two groups of organisms.

Applications of the Electron Microscope

Distinction between procaryotic and eucaryotic cells

The first task in identifying an unknown micro-organism would be to establish whether it is a procaryotic or eucaryotic cell. This can frequently be done by optical microscopy, but to be certain, especially in the case of some of the smaller unicellular forms, it is necessary to examine the cells in the electron microscope. The greatly increased resolution and magnification of the electron microscope can readily establish whether a cell is procaryotic or eucaryotic. For example, the thermophilic, acidophilic alga *Cyanidium caldarium* has been variously assigned to the blue-green algae, green algae, and the red algae. A detailed examination of the fine structure of this

organism by Rosen and Siegesmund (1961) clearly demonstrated a discrete nucleus and a simple chloroplast. On the basis of these structural findings and associated biochemical data on the photosynthetic pigments, this organism has now been placed more or less firmly in the red algae.

Distinction between algae and bacteria

The electron microscope has been useful to a lesser extent in establishing whether an organism is a blue-green alga or a bacterium. In most instances this is no problem, e.g. *Oscillatoria* is clearly a filamentous blue-green alga, while *Escherichia* is clearly a bacterium. The difficulty arises when one examines forms which have blue-green algal and bacterial characters. Thus the blue-green alga family of *Oscillatoriaceae* have characters in common with the bacterial families *Beggiatoaceae*, *Vitreoscillaceae*, and *Leucotrichaceae*, while the unicellular blue-green algae order of *Chroococcales* are similar in certain respects to some of the *Chlorobacteriaceae*. These and other such organisms are frequently colourless or lack the pigmentation usually associated with blue-green algae, and may live in the presence of hydrogen sulphide and possess sulphur granules. For a detailed description of such forms the reader is referred to Pringsheim (1963). Costerton, Murray and Robinson (1961) showed that certain strains of *Vitreoscilla* resembled bacteria in possessing a colourless cytoplasm and resembled blue-green algae in having a curious gliding motility. In comparing the fine structure of *Thioploca ingrica* and *Beggiatoa*, Maier and Murray (1965) were unable to decide whether these organisms were blue-green algae or bacteria.

Fine structure of photosynthetic apparatus

The fine structure of the photosynthetic apparatus is a criterion which may be useful in establishing the identity of unknown photoautotrophic procaryotes. The blue-green algae have peripherally located, complex photosynthetic lamellae, while the photosynthetic bacteria have less well-organized chromatophores or simple lamellae. (See Echlin and Morris, 1965, for detailed references to this.) It may thus be possible, solely on structural grounds, to consider as blue-green algae those organisms which possess peripheral photosynthetic lamellae, while conversely the absence of these structures would indicate that the organisms were bacteria. There are, however, problems associated with this proposed criterion, some of which may be difficult to resolve. It is not known, for example, whether colourless blue-green algae still possess photosynthetic lamellae. There are a few photosynthetic bacteria, such as species of *Rhodospirillum* and *Rhodomicrobium*, which possess fairly complex photosynthetic lamellae similar to

those seen in blue-green algae. The picture is complicated even further by the work of Murray and Watson (1965) on *Nitrocystis*, *Nitrobacter* and *Nitrosomonas*. These authors showed the presence of lamellae in these non-photosynthetic nitrifying bacteria. If, however, we accept the presence of peripheral photosynthetic lamellae together with the biochemical evidence of the presence of the c. phycobilins, as taxonomic indicators of the blue-green algae, this begins to delineate the group from other organisms.

Conditions of Culture and Specimen Preparation

Effect of variable conditions of culture

If we intend using criteria of fine structure as a means of identifying the blue-green algae, it will be necessary to rigidly standardize conditions of culture and specimen preparation. Echlin (1964) was able to show that the arrangement of photosynthetic lamellae in *Anacystis montana* grown in continuous illumination was dependent on the age of the organism. Younger cells tended to have the photosynthetic lamellae tightly appressed to each other, and as the cells matured, so the space between the lamellae was increased. Further work has suggested that the appearance of the blue-green algae varies according to the conditions under which the cells were grown. Variations such as whether the culture was agitated or stationary, the length, amount and type of illumination, the chemical nature of the culture medium, and whether the cells were grown in liquid or on solid medium may all affect the appearance of the organism and may well result in phenotypic variations. Fogg (1965) also considers some of the variations which result as a consequence of changes in the culture methods.

There is, however, some evidence that within certain broad limits the number of lamellae per cell does appear to be constant. "*Anacystis nidulans*" and *Pseudoholopedia* have relatively few (2–4) lamellae, while *Anacystis montana*, *Merismopedia glauca* and *Merismopedia punctata* have many (8–16) lamellae. Such figures were obtained from cells grown and fixed under the standard conditions enumerated later in this paper. This aspect of blue-green algal structure may be worth further examination, particuarly as Gibbs (1962) was able to demonstrate that the arrangement of the photosynthetic lamellae in higher algae showed certain group characteristics.

Methods of culture

The culture methods for blue-green algae have received considerable attention in the past, and as the algae are required for many different purposes, the methods vary between wide limits. Blue-green algae are cultured for four main purposes: (1) their maintenance in stock cultures;

(2) growth of algae for morphological and taxonomic studies; (3) the production of algae in such quantities as may be necessary for further study of their cytological, physiological and biochemical characteristics; and (4) the study of their growth characteristics. The culture methods for the latter two requirements are usually designed to elucidate specific problems—and it is not proposed to discuss these further. The reader is referred to the following references which record some of the specific methods for individual blue-green algae: Pringsheim (1946), Brunel, Prescott and Tiffany (1950), Allen (1952), Burlew (1953), Provasoli, McLaughlin and Droop (1957), Provasoli (1958), Provasoli and Pintner (1960), Chesnokoŭ (1961), Lewin (1961), Martin (1962), Myers (1962), and Starr (1964).

The present study is, however, concerned with the culture methods associated with the growth of algae for morphological and taxonomic studies, identification of blue-green algae, and to a lesser extent with the maintenance of stock cultures, which themselves provide inocula for further cultures. Blue-green algae are commonly isolated in, and subsequently maintained on, soil-water medium or on soil extract (Pringsheim, 1946), with the parameters of pH and ionic strength adjusted to be within the range of the natural habitat of the alga. Such cultures are unialgal, but are not usually bacteria-free. Once an organism is obtained in pure culture it is maintained either on the sterile soil extract or on solid media, consisting of a number of different media, to which 1% Difco Agar has been added, the choice of media being determined by the requirements of the organism. A list of such media may be found in the Appendix.

Algae for morphological studies should be on a medium which most closely approximates its natural habitat, and no contaminants, which may be indistinguishable from the organism under examination, should appear. In the absence of detailed culture methods optimal for whole orders of blue-green algae, it is suggested that the organisms should be grown in a shallow depth of soil-water medium, as this type of medium may be considered to most closely approximate that found in nature. The soil not only provides mineral nutrients and trace elements, but the latter are naturally chelated, the pH is buffered and toxic products rendered innocuous. It is also further suggested that the cultures should receive daylight and that the day-length remain unaltered by artificial illumination, though this general rule may require some modification depending on the natural habitat of the organism. The samples for examination in the electron microscope should be taken from the cultures from between two to four weeks old. By this time the cells will be in an exponential phase of growth. Sampling unicells for electron microscopy is difficult, because although the sample is initially large, when the material is seen in the microscope it is frequently at the one-cell level. It will, therefore, be necessary to examine a large number (50?) of cells in

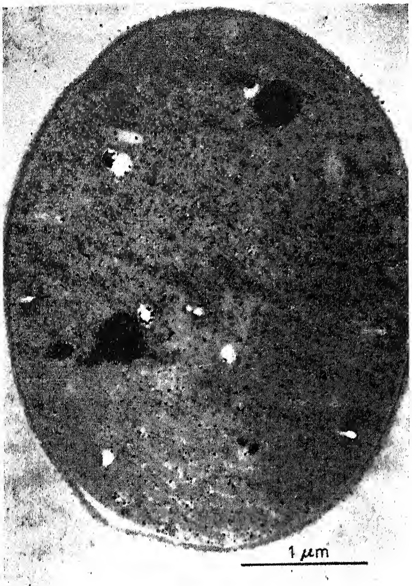

FIG. 1

FIG. 2

FIG. 1. Young cell of *Merismopedia glauca* fixed in glutaraldehyde and osmium tetroxide. × 34,000.

FIG. 2. Old cell of *Merismopedia glauca* fixed in glutaraldehyde and osmium tetroxide. × 33,000.

order to obtain a representative picture of the whole culture. For, although a culture as a whole may be in the exponential phase of growth, each individual cell as seen in the electron microscope may be young, old or senescent. In Figs 1, 2 and 3, micrographs of *Merismopedia glauca* may be seen, representing material from a young, old and a senescent culture respectively.

Methods of fixing

Having grown the cultures under relatively standard conditions, it is necessary to similarly prepare the material for examination in the electron microscope. Normally, when examining the ultrastructure of an unknown organism, it is frequently necessary to use a number of different fixatives in order to obtain a representative preservation of the tissue or cells. In this present study, one fixative should be used, and probably the most satisfactory general fixative would be 2% glutaraldehyde in phosphate buffer, pH 7·5, for about 30 min, followed by thorough washing in the same buffer and

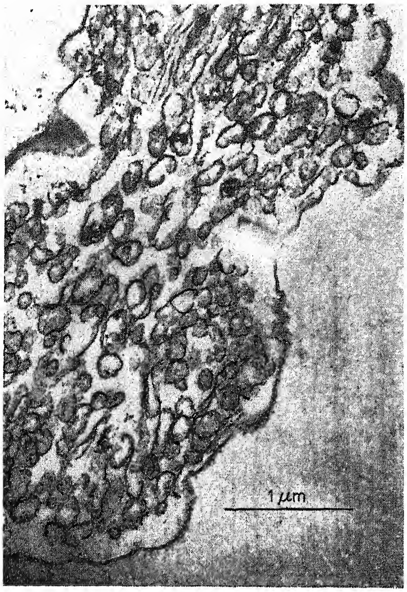

FIG. 3

FIG. 4

FIG. 3. Senescent cell of *Merismopedia glauca* fixed in glutaraldehyde and osmium tetroxide. × 36,000.

FIG. 4. Cell of *Merismopedia punctata* from exponential growth phase fixed in osmium tetroxide. × 29,000.

then a brief post-fixation in 1% osmium tetroxide in the same buffer for about 5 min. The cells may be blocked in agar, dehydrated and embedded in resin by one of the well-tried and accepted procedures (see, for example, Kay (1965)). Thin sections may then be cut on an ultramicrotome, "stained" with some heavy metal salt such as lead or uranium and examined in the electron microscope. The procedure gives adequate preservation, and reveals details of the nucleoplasm and the photosynthetic lamellae, the two criteria which would establish whether the cell was procaryotic in the first instance or a blue-green alga in the second.

Effect of variation in fixation

The consequences of variation in fixation may be seen in Figs 4–9. Cells of *Merismopedia punctata* and *Anacystis montana* from the same culture were fixed and stained by three different methods, and each reveals a strikingly different appearance of the same organism.

FIG. 5

FIG. 6

FIG. 5. Cell of *Merismopedia punctata* from exponential growth phase fixed in glutaraldehyde and osmium tetroxide. × 40,000.

FIG. 6. Cells of *Merismopedia punctata* from exponential growth phase fixed in permanganate. × 36,000.

Discussion and Conclusions

In spite of these rather elaborate preparations it is still practically impossible to distinguish between two closely related species of blue-green algae by means of electron micrographs alone. The only other criteria which may be used are the size and shape of the organism and the general appearance of growth. This may more easily be done by means of an optical microscopic examination of the different organisms grown on the same medium.

The new technique of scanning reflection electron microscopy may give additional information regarding the shape and size of unicellular and filamentous blue-green algae. This instrument, the Stereoscan, manufactured by the Cambridge Instrument Company, has been used in some preliminary studies on blue-green algae. The Stereoscan has a greatly increased utilizable depth of focus and gives an apparent three-dimensional picture. It has a limited resolution of about 2–300 Å, which is, however, one order of magnitude better than that obtained by conventional optical microscopes. Figs 10, 11 and 12 show some pictures of blue-green algae

FIG. 7

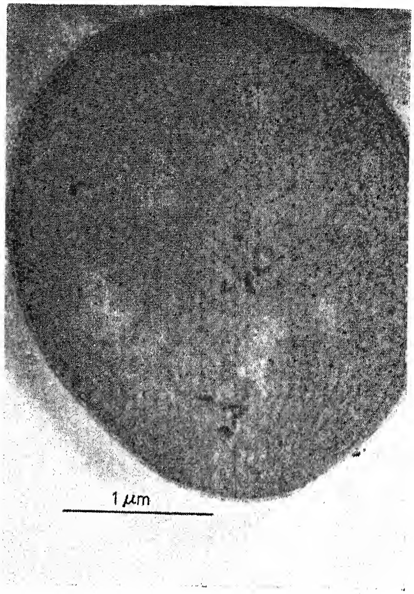

FIG. 9

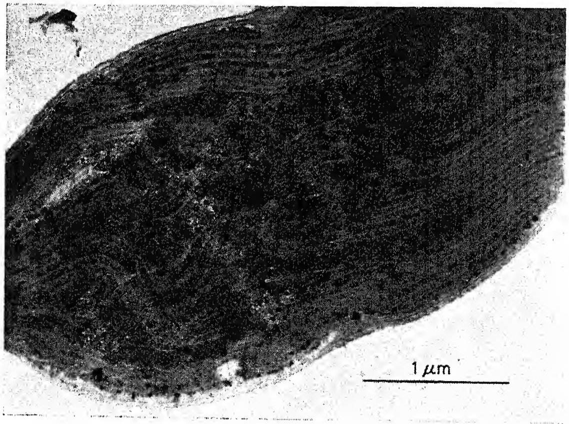

FIG. 8

FIG. 7. Cell of *Anacystis montana* from exponential growth phase fixed in osmium tetroxide. × 40,000.

FIG. 8. Cell of *Anacystis montana* from exponential growth phase fixed in glutaraldehyde and osmium tetroxide. × 40,000.

FIG. 9. Cell of *Anacystis montana* from exponential growth phase fixed in permanganate. × 46,000.

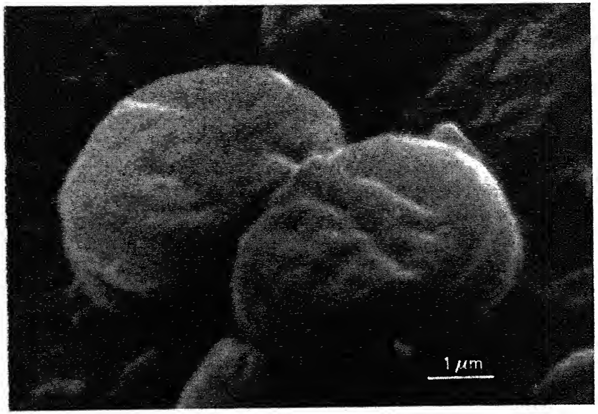

FIG. 10

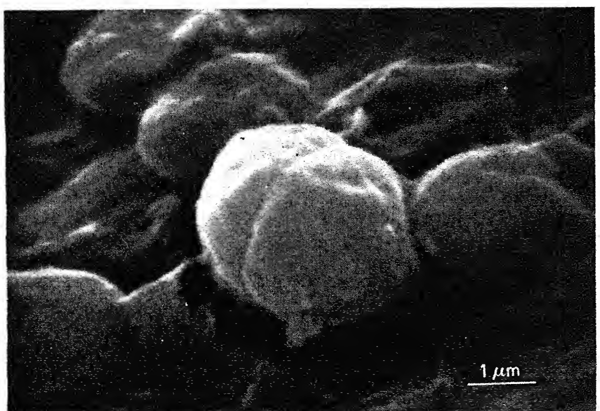

FIG. 11

FIG. 12

FIG. 10. Scanning reflection electron micrograph of *Merismopedia glauca*. × 18,600.

FIG. 11. Scanning reflection electron micrograph of *Anacystis montana*. × 18,600.

FIG. 12. Scanning reflection electron micrograph of *Oscillatoria formosa*. × 9800.

obtained by this technique, and while some problems still remain regarding the preservation of the material and the prevention of shrinkage, the micrographs do for the first time give some idea of the three-dimensional shape and surface topography of the smaller blue-green algae.

Mention must be made of three other techniques of electron microscopy, that of surface replicas, freeze etching and negative staining, which also provide valuable information regarding fine details of ultrastructure, particularly of cell-surface features.

In conclusion, it may be said that within certain defined limits the electron microscope can provide information which will help in the identification of unknown blue-green algae, particularly the smaller unicellular forms. It is imperative, however, that this ultrastructural information be used in conjunction with other criteria, particularly those obtained by optical microscopy and from biochemical and physiological studies.

The author is grateful to the Cambridge Instrument Company for the use of the "Stereoscan" Scanning Reflection Electron Microscope, and to Mr E. A. George, Curator of the Cambridge Collection of Algae and Protozoa, for valuable advice regarding the culture of blue-green algae.

APPENDIX

Culture Media for Blue-green Algae

The media given below are those successfully employed in the Cambridge Collection of Algae and Protozoa for the maintenance of different blue-green alga.

Cyanophycean Agar

Glass distilled water	100 ml
KNO_3	0·5%
K_2HPO_4	0·02%
$MgSO_4.7H_2O$	0·01%
Ferrous ammonium citrate	1 drop of 1% solution

Mastigocladus Agar

Distilled water	100 ml
$MgSO_4.7H_2O$	0·02%
K_2HPO_4	0·02%
$NaHCO_3$	0·02%
$CaCl_2$	0·01%
Soil extract	10%
Ferrous ammonium citrate	1·0 ml 0·1% solution

Phormidium Agar

Distilled water	100 ml
Soil extract	10%
KNO_3	0·02%
K_2HPO_4	0·002%
Sodium glutamate	0·05%
Glycine	0·05%
Dehydrated Liver infusion (OXOID)	0·002%
Trace element 0·1 ml stock solution*	
Artificial sea water (double strength)	36%
Pringsheim (1946)†	

Porphyridium Agar

Distilled water	100 ml
Soil extract	10%
Bacto-Yeast extract (DIFCO)	0·1%
Bacto-Tryptone (DIFCO)	0·1%
Artificial sea water (double strength)	33%
K_2HPO_4	0·002%
$MgSO_4 . 7H_2O$	0·002%
KNO_3	0·02%

E and S Agar

Distilled water	100 ml
K_2HPO_4	0·002%
$MgSO_4 . 7H_2O$	0·002%
KNO_3	0·02%
Soil extract	10%

Soil-water media

A layer about 1 cm deep of good calcareous loam is placed into a test-tube. Carefully add 7–10 cm of water, plug or cover, and steam for one hour (longer for larger vessels) on each of two consecutive days. Allow to stand for a further day before inoculating, when the pH should be between 7 and 8.

* Trace Element Stock Solution. A litre contains: $Na_2B_2O_7 . 10H_2O$, 20 mg; $CaSO_4 . 5H_2O$, 100 mg; Fe_3PO_4, 150 mg; $MnSO_4 . 4H_2O$, 20 mg; $Na_2M_0O_4 . 2H_2O$, 10 mg; Zn $SO_4 . 7H_2O$, 100 mg; in distilled water. It may be found necessary to adjust the make up of the Trace Element Solutions to suit the particular requirements of the blue-green alga under investigation. However, most blue-green algae appear to show optimal growth when the above trace elements stock solution is used, or the trace element solution suggested by Allen and Arnon (1955).

† There is a misprint in this book, the KCl concentration should be 0·075% *not* 1%.

Soil-extract Solution

The soil-extract solution is made by heating in a steamer a calcareous garden loam with twice its volume of supernatant water.

Autoclaving

The above media are autoclaved at 121° for 3 min at a pressure of 15 lb/in^2. It is generally felt that a minimum time for autoclaving is more desirable than the more conventional 15 lb/15 min employed in bacteriological laboratories. Experience has shown that the short autoclaving destroys all spores likely to be present, and contaminated media is only very rarely experienced. Longer autoclaving has been found to result in degradative changes in the media, and the resulting solutions do not permit the optimal growth of the organisms.

In those media employing ferrous ammonium citrate, this solution is made up and autoclaved separately, and added to the final media. Alternatively the iron may be chelated with EDTA, in which case there is no need to autoclave this separately.

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Two Simplified Schemes for Identifying Yeast Cultures

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Most major groups of organisms are recognizable by certain well-defined characteristics. Yeasts, because of their wide spectrum of morphological and biochemical properties, do not comply with any one agreed formula. Perhaps their only definition is that they are a non-homogeneous group of fungi, placed there arbitrarily by acknowledged experts (Lodder, Sloof and Kreger-van Rij, 1958). The division of yeasts into the major subgroups of families and genera is based mainly on mycological tests, and into species as a result of tests derived largely from bacteriology. While the requirements for classifying and identifying an organism are different (Cowan, 1965), the heterogeneity of the group and the diversity of tests required make the problem of identifying a yeast culture unnecessarily complex. Successful identification of a yeast, particularly by anyone unfamiliar with the specialized forms of mycelia and pseudomycelia, demands tests that give unequivocal results, as far as that is ever possible in microbiology. Such morphological characteristics as the method of vegetative reproduction, the formation of pigment and polysaccharide are stable and, therefore, suitable. On the other hand, spore formation is difficult to induce in practice. Consequently, sporulation, of primary importance in classification, is not used in the identification schemes described below, where pairs of perfect and imperfect forms are considered to be identical. In contrast, the results of such biochemical tests as production of gas from sugars or their assimilation (utilization), utilization of nitrate, etc., are of major importance for identification purposes. These tests can be used by anyone with some training in general microbiological techniques and the results are reproducible in different laboratories, provided the operating conditions are carefully defined and rigidly observed.

Two schemes for identifying yeast cultures are outlined below. In the first, the Morphological Key, primary separation is based on colonial appearance. This is particularly valuable when large numbers of yeasts are to be split into small groups with similar properties, as in ecological surveys. The second, or Biochemical Key, is suitable for smaller numbers of cultures, as might be obtained in tracing a particular yeast through a manufacturing process (Goswell and Burnett, 1966).

The yeasts listed in both keys are those described in Wickerham (1951), Lodder and Kreger-van Rij (1952) and Kreger-van Rij (1964). References to cultures isolated between 1952 and 1965 may be found in Soyugenc and Hedrick (1966).

Materials and Methods

Incubation

Normally all cultures are incubated aerobically at 25°, but 15–17° should be used for carotenoid-forming or psychrophilic yeasts.

Morphology

Colonial appearance, pigment and polysaccharide formation are observed by streaking the culture on Difco Morphology Agar, Oxoid Malt Wort Agar or MYPG Agar (Wickerham, 1951), reinforced with 0·05% ferric ammonium citrate and 2μg biotin/l. Incubation is for 5 days initially.

Pellicle formation is observed by inoculating a loopful of culture into the liquid form of one of the above media, dispensed in 30 ml lots per 6 oz screw-mouth bottle, and incubating for 5 days.

For pseudomycelium formation (Dalmau, 1929; Wickerham, 1951), melt 1·7% sterile Difco Cornmeal Agar, pour into Petri dishes and dry at room temperature for 2 days (Beech, 1957), before streaking with up to 5 cultures per plate: place a sterile cover slip over part of each streak. After 3–5 days' incubation pseudomycelium formation can be observed in the aerobic or anaerobic portions of the streak.

Fermentation

The basal medium, 0·45% Difco Yeast Extract and 0·75% peptone (Allen and Hanbury Eupeptone or Difco Bacto Peptone), is adjusted to pH 6·4 and enough Brom-thymol-Blue added to give an intense blue colour (Wickerham, 1951). Dispense 4 ml lots in 150 × 15 mm test tubes, each with an inverted 50 × 10 mm Durham tube. Sterilize at 121° for 15 min and, when cool, add to different tubes 2 ml of the sterile-filtered 6% solutions of the sugars, glucose, galactose, sucrose, maltose and lactose, but 8% meli-

biose and 12% raffinose. Inoculate into each tube a loopful of yeast or 0·1 ml of a standardized washed inoculum. Observe daily for gas formation.

Assimilation of nitrate

Add 0·5 ml of a sterile-filtered solution of 11·7% Difco Carbon Base and 0·78% potassium nitrate to 4·5 ml sterile water. Inoculate a tube with 0·2 ml washed inoculum; after 7 days inoculate a second tube with 0·1 ml from the first. Assess turbidity after a further 7 days' incubation (Wickerham, 1946).

Assimilation of sugars

Dispense 5 ml of a sterile-filtered solution of 6·7% Difco Yeast Nitrogen Base and 5% of the sugar being tested, as for the nitrate test. First incubate the culture for 4 days in this medium, but using only one-tenth of the sugar concentration. Prepare, using the method of Wickerham (1951), a washed standardized suspension from this low sugar medium and inoculate 0·1 ml into each of the sugar assimilation tubes. Estimate turbidity at weekly intervals for one month.

Starch production

9 ml of 3% glucose solution in a 100 ml conical flask are brought up to 121° in the autoclave and the source of heat shut off (Davies, 1939). 1 ml of sterile-filtered 6·7% Difco Yeast Nitrogen Base solution is added when the flasks cool. Two flasks are inoculated for each yeast and tested with iodine for starch after 7 and 14 days incubation, respectively.

Acid production (Custers, 1940)

5% glucose, 0·5% reprecipitated $CaCO_3$, 2% Difco Yeast Extract, and 2% agar are dispensed in 6 oz screw-mouth bottles and sterilized at 115° for 15 min. Cool to 50°, mix thoroughly, slant in sterile test-tubes that are plunged immediately into an ice bath. The culture is streaked down the centre of the slant and acid production observed during incubation by the dissolution of the chalk.

Pigment formation

Coloured deposits in the glucose assimilation tubes are separated by centrifugation. If the pigment is soluble in acetone it is a carotenoid; whereas solubility in 5% ethanolic KOH indicates pulcherrimin. Carotenoid production can be confirmed by growing the culture in 100 ml of the same medium and using the test of Peterson, Bell, Etchells and Smart (1954). Pulcherrimin can be confirmed by the method of van der Walt (1952) or Cook and Slater (1954).

Osmophilic yeast medium

Yeasts able to grow on 60% w/w glucose, 1% Difco Yeast Extract and 2% agar after 7 days' at 30° are considered to be osmophilic (Lodder and Kreger-van Rij, 1952).

Morphological Key

Yeasts isolated only rarely or found in very specialized environments have been ignored, e.g. those in the subfamily Nematospora and the genera *Bullera*, *Endomyces*, *Endomycopsis*, *Nadsonia*, *Pityrosporum*, *Schizosaccharomyces*, *Schwanniomyces* and *Trigonopsis*. The gross appearance of the colonies and method of vegetative budding are required, but not spore formation, specialized forms of pseudomycelia, capsules and other cytological detail. Cell size and shape are needed only for extreme forms.

The culture is first streaked on the morphology agar, incubated for 5 days' and examined under a plate microscope. Better colonial differentiation will be obtained on a medium related to the original source of the yeast, where this is known. For example, fruit juices will need the addition of 1% Difco Yeast Extract, pH adjustment to 4·8 and to be solidified with 3% agar (Beech, 1953). Incubation should be continued for a further 5 days' if colonies fail to form.

The culture is first examined for purity. Should two distinct colonial forms be present, it may be due to the presence and absence of spores as in *Citeromyces* spp., or to haploid and diploid cells being present in the same culture. Microscopic examination of wet preparations will be needed to check these points. The colonies can then be described as pigmented, mucoid, rough, smooth, or slow-growing for late-developing yeasts, as outlined in Table 1.

TABLE 1. Primary separation of yeasts on colonial appearance

Days to form colonies	Group	Appearance of colony
5	Pigmented	Colour ranging from shades of buff through red to black
	Non-pigmented	
	Mucoid	Convex becoming spreading and amoeboid, runny consistency, smooth and glistening
	Smooth	Completely smooth surface, no projections, uniform texture, shiny/dull
	Rough	Convex rugose and convex papillate, cratoriform, surface showing striations, contours, wrinkles, powdery
10	Slow-growing	Cauliflower or small convex, often irregular

TABLE 2. Differentiation of pigmented "yeasts"

Buff, ginger, grey, black and green colonies	
Juvenile colonies lighter coloured	
Mature colonies black or green	*Aureobasidium* spp.
Pseudomycelial edges	*Trichosporon* spp.
Black colonies	
No pseudomycelial edges	*Cladosporium* spp.
Yellow, orange, pink, red and maroon colonies	
Carotenoid:	
Pigment soluble in organic solvents	*Sporobolomyces* spp.
Non-fermenting	*Rhodotorula* spp.
Pulcherrimin:	
Pigment soluble in ethanolic KOH	*Saccharomyces lactis*
Fermenting yeasts	*S. fragilis*
Lactose assimilated	*Candida pseudotropicalis*
	S. marxianus
	C. macedoniensis
Lactose not assimilated	*C. pulcherrima*

Pigmented yeast colonies can be classed as "black" or red (Table 2). While Lodder, Sloof and Kreger-van Rij (1958) admit the existence in nature of dark-pigmented organisms analogous to non- or light-pigmented yeasts, they consider that only the *Trichosporon* spp. from the former group should be classed as true yeasts. Mrak and Phaff (1948) would also include *Aureobasidium* (*Pullularia*) *pullulans*: this species and *Cladosporium* spp. are often found in the same environment as yeasts, and can cause confusion during isolation. Carotenoid-forming yeasts (optimum 15–17°), include species from the genera *Sporobolomyces* and *Rhodotorula*. The former easily lose their ability to form ballistospores on laboratory cultivation, so that species of the two genera become indistinguishable; three such pairs are shown in Table 3.

The remaining pigmented yeasts produce pulcherrimin in the presence of iron. This is particularly marked with the non-lactose-assimilating yeast *Candida pulcherrima*, which in high concentrations of iron binds the pigment strongly in its cells, but in low concentrations allows it to diffuse into the medium. The lactose assimilating yeasts only produce small pigmented colonies on nutritionally deficient media. They are, therefore, included both in this section and in Table 5. They were once included in a proposed new genus, *Dekkeromyces* (Wickerham, 1955; Wickerham and Burton, 1956*a*, *b*), which was not, however, clearly defined. Subsequently, van der Walt (1965) has validly published the name *Kluyveromyces* for this group of yeasts.

TABLE 3. Separation of carotenoid-forming yeasts into species-groups

Assimilation patterns: Nitrate	Sugars	
Positive	Glucose, sucrose, maltose	*Sporobolomyces roseus*
	Glucose, galactose, sucrose, maltose	*Rhodotorula glutinis* (oval cells) *R. aurantiaca* (elongated cells)
	Glucose, sucrose	*S. salmonicolor* (forms mycelium) *S. odorus* (no mycelium)
Negative	Glucose	*S. gracilis* *R. pallida*
	Glucose, sucrose, maltose	*S. pararoseus* *R. mucilaginosa*
	Glucose, galactose, sucrose, maltose	*S. alborubescens* *R. rubra*
	Glucose, galactose, sucrose	*R. minuta*
	Glucose, galactose, sucrose, maltose, lactose	*R. flava*

Saccharomyces lactis and *S. fragilis* ferment lactose, but *S. marxianus* does not: *S. lactis* assimilates maltose, but *S. fragilis* does not. The imperfect form of *S. lactis*, *Torulopsis sphaerica*, does not form pulcherrimin (van der Walt, 1952), whereas the other two imperfect yeasts behave like their sporing partners. The addition of biotin to the medium ensures that adenine-independent cultures of *S. cerevisiae* and *Schizosaccharomyces octosporus* do not produce a pink pigment (Cutts and Rainbow, 1950; Chamberlain, Cutts and Rainbow, 1952; Chamberlain and Rainbow, 1954). Certain mutants of *S. cerevisiae* can produce a pigment, independent of the iron or biotin concentration in the medium (Lindegren and Lindegren, 1947; Ephrussi, Hottinguer and Tavlitzki, 1949; Winge and Roberts, 1950), but these do not occur naturally.

The differentiation of yeasts producing non-pigmented colonies is shown in Tables 4 and 5. Glistening, spreading, almost amoeboid colonies are due to yeasts that form large amounts of polysaccharide. In this group of "mucoid" yeasts, some give the characteristic blue colour of amylose (Hehre, Carlson and Hamilton, 1949) when iodine is added to the culture

TABLE 4. Separation of non-pigmented yeasts

Days to form colonies	Colony type		
5	Mucoid	Produce starch	Starch not produced
		Nitrate positive	Glucose fermented
		Cryptococcus albidus	*Torulopsis molischiana*
		Crypt. diffluens	*T. pinus*
		Nitrate negative	Glucose not fermented
		Crypt. laurentii	*Lipomyces starkeyi*
		Crypt. neoformans	*L. lipoferus*
		Crypt. luteolus	
		Candida humicola	
		Candida curvata	
	Smooth Rough	} See Tables 5 and 6	
10	Cauliflower shape or small and conical Turn brown as acid formed	Apiculate cells *Kloeckera* spp.	Ogive cells *Brettanomyces anomalus* *B. lambicus* } *B. bruxellensis* } *B. claussenii*

medium. These yeasts can be further subdivided by their ability or inability to assimilate nitrate as sole source of nitrogen. Of those that do, *Cryptococcus albidus* assimilates lactose, but not *Crypt. diffluens*; in the second group, *Crypt. laurentii*, *Candida humicola* and *C. curvata* assimilate lactose. The nitrate-negative, lactose-assimilating yeasts are difficult to separate. All three form pellicles; *C. humicola* forms a true mycelium, but the other two show pseudomycelia of varying complexity. Similarly differentiation of the lactose-negative yeasts is dependent on the possible formation of a pellicle and/or cell shape. The polysaccharide-producing yeasts can be subdivided on their ability to ferment glucose. *T. molischiana* assimilates maltose, but not *T. pinus*. The non-fermenting *Lipomyces* spp. are separated on the assimilation of lactose, being positive for *L. lipoferus*.

The next most obvious group are the slow-growing yeasts that never form large colonies, even on prolonged incubation, and which invariably become brown due to acid formation. We have never been able to characterize the lemon-cell-shaped *Kloeckera* spp. (Beech, 1957; Bowen, 1962), because they never survived purification. *Brettanomyces anomalus* and *B.*

claussenii are separated from the other two species by their positive fermentation of galactose. *B. claussenii* can ferment sucrose and maltose, which *B. anomalus* can not. *B. lambicus* and *B. bruxellensis* are only separated with difficulty (Lodder and Kreger-van Rij, 1952), by differences in the surface appearance of their colonies and pellicles. *Torulopsis anomala*, *T. versatilis* and *B. claussenii* are the exceptions to the third of the Kluyver–Dekker laws (Kluyver, 1931), in that they ferment both maltose and lactose. The first two yeasts and *T. etchellsii* are considered by Etchells and Bell (1950) and by Wickerham (1952) to belong to the genus *Brettanomyces*.

Species forming rough and/or smooth, non-pigmented, non-mucoid colonies, can be further separated on morphological appearance, but considerable experience with yeasts is necessary before this can be done with any degree of certainty. It is better, therefore, to complete any remaining differentiation using biochemical tests. A preliminary separation is shown in Table 5; yeasts in the four groups can be separated further by assimilation of the five standard sugars, of other carbon compounds, and by growth on high osmotic pressure media, etc. Groups based on these criteria are given in Table 6.

Biochemical Key

There are two ways of carrying out biochemical tests. The first is to use a standard method, irrespective of its sensitivity, incubate for *exactly* the stated time, and to record any reaction as Positive. This is the basis used for Table 5. The second is to use the most sensitive method, prolong incubation for a month and attempt to differentiate between + and Weak, and between Weak and Latent. It is not always clear from the literature which method has been used and yeasts may be recorded as, for example, "fermentation of glucose, Positive, sometimes Weak, occasionally Negative". Table 6 has been built up from published data, but to ensure that such yeasts are correctly identified they will be found in several groups, so that all possible combinations of results have been covered. Because of these different criteria, the groupings of yeasts in Tables 5 and 6 may be different.

All the yeasts examined in the Morphological Key are included here, with the addition of *Schizosaccharomyces* spp.

In the Biochemical Key, yeasts are divided primarily on their fermentation and assimilation patterns, and morphological details are only of secondary importance. Further, although the usual binomial names are given to the yeasts, they could equally be given a coded description based on these patterns. This can only be done economically if grouped characters are used in different positions in a sequential code (Cowan, 1965). To this end the theoretical possibilities of the code will be outlined in the different sections of the Key.

TABLE 5. Separation of non-pigmented, non-mucoid yeast species

Positive nitrate assimilation Non-fermenting yeasts	 Fermenting yeasts
Galactose assimilated *C. scottii* (cylindrical cells) *T. aeria* (round cells)	Glucose fermented only *Hansenula capsulata* *H. minuta* *H. angusta* *H. mrakii* *H. silvicola* *T. etchellsii* (osmophile) *T. magnoliae*
Galactose not assimilated *C. melinii* *H. beckii* *H. canadensis* *H. californica*	Glucose and maltose assimilated *H. ciferri* *H. schneggii*
	Glucose, galactose and maltose assimilated *T. anomala*
	Glucose, sucrose and raffinose assimilated *H. suaveolens* *H. jadinii* *H. saturnus* *H. subpelliculosa* *H. anomala* *C. utilis* *C. pelliculosa* *T. globosa* (osmophile) *T. lactis-condensii*
	Glucose, galactose, sucrose, maltose and raffinose fermented *T. versatilis* (osmophile)

Negative nitrate assimilation Non-fermenting yeasts	
Glucose assimilated only *C. mycoderma* *Pichia membranaefaciens* *C. zeylanoides* *C. lipolytica* *P. fluxuum* *P. terricola* *P. pinus* *P. quercuum* *P. salictaria*	Glucose and galactose assimilated *P. haplophila* *P. chambardii* Glucose, sucrose and maltose assimilated *P. toletana* *T. ernobii* *C. mesenterica* *C. japonica*

T. inconspicua
(*T. pinus*—if not mucoid)

Glucose, galactose, sucrose, maltose and lactose assimilated
C. humicola } if not
C. curvata } mucoid
P. scolyti
P. polymorpha
P. pseudopolymorpha

Glucose, galactose, sucrose and lactose assimilated
Debaromyces tamarii

Glucose, galactose and sucrose assimilated
T. gropengiesseri

Glucose, galactose, sucrose and maltose assimilated
C. reukaufii
P. etchellsii
P. vini (formerly *Deb. vini*)
Deb. hansenii }
Deb. kloeckeri }
T. famata }
Deb. vanriji
Deb. marama
T. candida

Glucose, galactose and maltose assimilated
C. brumptii
Deb. coudertii

Negative nitrate assimilation
Glucose fermented only

Glucose assimilated only
Hanseniaspora (*P.*) *pijperi* (bipolar)
Hanseniaspora valbyensis (bipolar)
K. apiculata (bipolar)
S. bisporus
S. pastori——→*P. pastoris*
S. baillii
S. acidifaciens (acid producer)
S. mellis (osmophile)
S. elegans
P. saito
P. fermentans
P. trehalophila
P. orientalis
P. kluyveri
T. glabrata
C. krusei

Glucose and galactose assimilated
C. catenulata
P. farinosa

Glucose and maltose assimilated
S. rouxii (osmophile)
(*T. molischiana*—if not mucoid)
K. africana
K. corticis
K. magna

Glucose, galactose and sucrose assimilated
T. magnoliae

Glucose, sucrose and maltose assimilated
C. solani
P. rhodanensis
P. bovis
P. wickerhamii

Glucose, galactose, sucrose and maltose assimilated
C. parapsilosis
T. sake

Glucose and galactose fermented
C. tenuis
S. delbreukii

Glucose and maltose fermented
C. albicans
C. stellatoidea

Glucose and sucrose fermented
K. lafarii (bipolar)

Glucose, sucrose and maltose fermented
S. heterogenicus

Glucose, galactose and maltose fermented
S. italicus
C. claussenii

Negative nitrate assimilation
Fermenting yeasts

Glucose, sucrose and raffinose one-third fermented
C. guilliermondii }
P. guilliermondii }
P. strassburgensis
P. ohmeri
Deb. globosus
Deb. cantarellii
Deb. phaffii
Deb. castellii
T. stellata
T. dattila
T. bacillaris (osmophile)
S. veronae
S. rosei
S. chevalieri
S. marxianus }
C. macedoniensis }
K. javanica (bipolar)
K. jensenii (bipolar)
K. antillarum (bipolar)
S. microellipsodes (3/3rds)
C. melibiosi (2/3rds)

Glucose, galactose, sucrose and raffinose one-third fermented
S. exiguus }
T. holmii }
S. fructuum

Glucose, galactose, sucrose and maltose fermented
S. steineri
C. tropicalis

Glucose, sucrose, maltose and raffinose one-third fermented
P. robertsii
S. pastorianus (2/3rds)
S. oviformis
S. fermentati
S. bayanus
S. willianus
T. colliculosa

Glucose, galactose, sucrose, maltose and raffinose fermented
C. intermedia (1/3rd)
C. robusta } (1/3rd)
S. cerevisiae } (1/3rd)
S. florentinus (3/3rds)
S. carlsbergensis (3/3rds)
S. uvarum (3/3rds)
S. logos (3/3rds)
D. franciscae (osmophile) (3/3rds)

Glucose, galactose, sucrose, lactose and raffinose one-third fermented
S. lactis }
T. sphaerica }
S. fragilis }
C. pseudotropicalis }

TABLE 6. Biochemical key for indentifying yeast cultures

Sequential order of code numbers
Sugar fermentation pattern / Assimilation of nitrate / Sugar assimilation pattern / Morphology

Sugar fermentation pattern

O	Glucose not fermented
D	Glucose only fermented
G	Glucose and galactose fermented
M	Glucose and maltose fermented
S	Glucose and sucrose fermented
MS	Glucose, maltose and sucrose fermented
A	Glucose, sucrose and raffinose fermented
MA	Glucose, maltose, sucrose and raffinose fermented
MB	Glucose, maltose, sucrose, raffinose and melibiose fermented
B	Glucose, sucrose, raffinose and melibiose fermented
GM	Glucose, galactose and maltose fermented
GS	Glucose, galactose and sucrose fermented
GA	Glucose, galactose, sucrose and raffinose fermented
GB	Glucose, galactose, sucrose, raffinose and melibiose fermented
GMS	Glucose, galactose, maltose and sucrose fermented
GMA	Glucose, galactose, maltose, sucrose and raffinose fermented
GMB	Glucose, galactose, maltose, sucrose, raffinose and melibiose fermented
GL	Glucose, galactose and lactose fermented
GML	Glucose, galactose, maltose and lactose fermented
GAL	Glucose, galactose, sucrose, raffinose and lactose fermented
GMAL	Glucose, galactose, maltose, sucrose, raffinose and lactose fermented

Assimilation of nitrate

N = Assimilate potasium nitrate as sole source of nitrogen
O = Cannot utilize nitrate

Sugar assimilation pattern

1	Glucose assimilated only
1M	Glucose and maltose assimilated
1ML	Glucose, maltose and lactose assimilated
1L	Glucose and lactose assimilated
1S	Glucose and sucrose assimilated
1SL	Glucose, sucrose and lactose assimilated
1SM	Glucose, sucrose and maltose assimilated
1SML	Glucose, sucrose, maltose and lactose assimilated
2	Glucose and galactose assimilated
2M	Glucose, galactose and maltose assimilated
2ML	Glucose, galactose, maltose and lactose assimilated
2L	Glucose, galactose and lactose assimilated
3	Glucose, galactose and sucrose assimilated
3L	Glucose, galactose, sucrose and lactose assimilated
4	Glucose, galactose, sucrose and maltose assimilated
5	All sugars assimilated except raffinose

Morphology

FF	Fission yeast
BB	Bipolar budding
TRI	Triangular budding
1	Multilateral budding, no pseudomycelium, no pellicle
2	Multilateral budding, with pseudomycelium, with pellicle
2A	Multilateral budding, no pseudomycelium, with pellicle
2B	Multilateral budding, with pseudomycelium, no pellicle
CAPS	Capsule formed
COL	Colour in streak

Abbreviations used in the tables

B. = *Brettanomyces*
Bu. = *Bullera*
C. = *Candida*
Crypt. = *Cryptococcus*
Deb. = *Debaryomyces*
H. = *Hansenula*
K. = *Kloeckera*
L. = *Lipomyces*
P. = *Pichia*
R. = *Rhodotorula*
S. = *Saccharomyces*
Sp. = *Sporobolomyces*
T. = *Torulopsis*
Tr. = *Trigonopsis*

O/O1	/1	*T. inconspicua* (may ferment glucose very weakly) *T. pinus*
	/COL	*R. pallida* *Sp. gracilis*
	/2	*P. membranaefaciens* (*P. terricola* (*P. quercuum* } ferments glucose weakly) *P. fluxuum* *C. mycoderma* *C. lipolytica*
	/2A	*P. membranaefaciens* (*P. terricola* (*P. quercuum* } ferments glucose weakly) *P. fluxuum* *P. salictaria*
	/2B	*C. zeylanoides* (*P. quercuum*, ferments glucose weakly)
O/O2	/COL	*R. pallida*

O/O2	/2	*C. rugosa* *P. haplophila*
	/2A	*P. chambardii* *P. haplophila*
	/TRI	*Tr. variablis*
O/O3	/1	(*T. gropengiesseri*, ferments glucose and sucrose)
	/COL	*R. minuta*
O/O4	/1	*T. famata* *Deb. hansenii* *Deb. marama*
	/2	*Deb. vanriji* *P. vini*
	/2A	*Deb. hansenii* *Deb. marama* (*T. candida*, weak lactose assimilation) *P. etchellsii*
	/CAPS	*L. starkeyi* *Crypt. neoformans* *Crypt. luteolus*
	/COL	*R. rubra* *Sp. albo-rubescens* *Sp. pararoseus*
O/O5	/1	(*T. candida*) (*Deb. hansenii*)
	/2	*P. pseudopolymorpha* *P. polymorpha* *C. humicola* } oval cylindrical cells *C. curvata* } *T. candida* } small short oval cells *Deb. hansenii* }
	/COL	*R. flava*
	/CAPS	*L. lipoferus* *Bu. alba*
	/CAPS	*Crypt. laurentii*

O/O1SML	/CAPS	*Crypt. laurentii*
O/O1SM	/1	*T. ernobii*
	/COL	*R. mucilaginosa* *Sp. pararoseus*
	/2	*C. mesenterica*
	/2B	*C. mesenterica* *C. japonica* (*P. toletana*, ferments glucose weakly)
	/CAPS	*Crypt. neoformans*
O/O2M	/1 /2	*Deb. coudertii* *C. brumptii*
O/O3L	/1	*Deb. tamarii* (very slow fermentation of glucose, galactose, sucrose and/or lactose)
O/N5	/1 /CAPS	*T. aeria* *Crypt. albidus*
O/N4	/1	*T. aeria*
	/COL	*R. glutinis* *R. aurantiaca* *Sp. roseus*
	/CAPS	*Crypt. diffluens*
	/2B	*C. scottii*
O/N1SM	/2B	*C. melinii*
	/CAPS	(*Crypt. albidus*, weak galactose and lactose fermentation) (*Crypt. diffluens*, weak galactose fermentation)
	/COL	*Sp. roseus*
O/N1SML O/N5	/CAPS	(*Crypt. albidus*, weak galactose assimilation)
O/N3	/COL	*Sp. salmonicolor*
O/NIS	/COL	*Sp. odorus*
D/O1	/1	*P. pinus* *P. pastoris* *T. inconspicua* (ferments glucose very weakly)

D/O1		*P. trehalophila* *T. glabrata* *S. mellis* (osmophile) *S. elegans* (may ferment sucrose weakly) *S. acidifaciens* (acid-former)
	/2	*P. pijperi* (*Hanseniaspora pijperi*) *P. fermentans* *P. saito* *P. kluyveri* *P. terricola* *P. orientalis* *C. krusei* *C. mycoderma*
	/2B	*S. bailii* *S. acidifaciens*
	/BB	*K. apiculata* *Hanseniaspora valbyensis*
D/O2	/1	*S. bisporus* *S. mellis* (osmophile) *S. acidifaciens* (*S. delbrueckii*, ferments galactose weakly)
	/2	*P. farinosa* (*C. catenulata*, ferments galactose weakly)
D/O3	/1	(*S. elegans*, ferments sucrose slowly and raffinose weakly) (*T. gropengiesseri*, ferments sucrose weakly) (*T. magnoliae*, ferments sucrose weakly)
D/O4	/1	*T. famata*
	/2	*C. reukaufii* (*C. solani*, ferments sucrose weakly) (*C. parapsilosis* ferments galactose weakly) *C. pulcherrima*
	/2B	*P. etchellsii*
	/COL	*C. pulcherrima*
D/O5	/1	*T. candida*
	/2	(*P. polymorpha*, weak sucrose fermentation)
	/2B	(*P. scolyti*, weak galactose fermentation)
D/O1M	/1	*T. molischiana*
	/1 or 2	*S. rouxii* (ferments maltose weakly—osmophile)
	/BB	*K. corticus* *K. magna*

D/O1SM	/1	*T. ernobii*
	/2	*P. wickerhamii* *P. rhodanensis* *P. toletana*
	/2A	*P. bovis*
	/COL	*C. pulcherrima*
	/2	*C. solani* (ferments sucrose weakly)
	/BB	*K. africana*
D/O2M	/2	*C. brumptii*
D/NI	/1	*H. minuta* (*T. etchellsii*, ferments maltose weakly)
	/2 or 2A	*H. mrakii*
D/N4	/2B	*H. silvicola* (ferments galactose weakly)
D/N3	/1	*T. magnoliae* (ferments sucrose weakly)
D/N1S	/2A	*H. californica*
G/O2	/1	*S. delbrueckii*
	/2	*P. farinosa* *C. catenulata*
	/2B	*Trichosporon fermentans*
G/O4	/1	(*T. sake*, ferments sucrose and maltose weakly)
	/2	*C. pulcherrima*
	/2 or 2B	*C. parapsilosis*
	/2B	(*C. tenuis*, weak lactose assimilation)
	/COL	*C. pulcherrima*
G/O5	/2B	*C. tenuis*
G/O1SM	/2 /COL	*C. pulcherrima*

G/N4	/2B	*H. silvicola*
M/O4	/2B	(*C. albicans*, weak galactose fermentation)
M/O1M	/FF	*Schizosaccharomyces octosporus*
M/O2M	/1	*S. rouxii* (osmophile)
	/2B	*C. stellatoidea*
M/N1 N2 N1M N2M	/1	*T. etchellsii*
M/N4	/2	(*H. schneggii*, weak glucose and sucrose fermentation)
S/O3	/1	*T. gropengiesseri* *T. magnoliae*
S/O4	/1	(*T. candida*, lactose assimilated weakly)
	/2A	(*T. candida*, lactose assimilated weakly) (*T. sake*, galactose and maltose fermented weakly)
	/2B	(*Trichosporon behrendii*, galactose and maltose fermented weakly. Large abnormal cells) *C. solani*
S/O5	/1	*T. candida* *Deb. hansenii*
	/2	*P. pseudopolymorpha* *P. polymorpha*
	/2A	*Deb. hansenii*
	/2B	*T. candida*
S/O1S	/BB	*K. lafarii*
S/O1SM	/2	*P. rhodanensis*
	/2B	*C. solani*
MA/O4	/1 or 2 or 2B	*S. cerevisiae*
MS/O1SM	/2B	*S. heterogenicus*

MS/N4	/2	*B. lambicus* *B. bruxellensis* (*H. schnegii*, ferments galactose weakly)
MS/N1SM	/2	*B. bruxellensis*
A/O1 O2	/1	*S. elegans*
A/O3	/1	*S. elegans*
	/2	*S. chevalieri*
	/2B	*S. elegans*
A/O1S	/1	*Deb. globosus* *S. rosei* *T. stellata* *T. bacillaris* *S. elegans*
	/2B	*S. elegans*
	/BB	*Saccharomycodes ludwigii* *K. javanica* *K. antillarum* *K. jensennii*
A/O4	/1	*T. dattila*
	/2	*P. ohmeri* *C. guillermondii* *P. guillermondii*
	/2B	*P. strassburgensis* *S. veronae*
	/2A	*Deb. phaffii*
A/O5	/2	*P. polymorpha* *P. pseudopolymorpha*
	/2A	*Deb. castellii* *Deb. cantarellii*
A/N4	/2	*H. subpelliculosa*
A/NIS	/1	*T. lactis-condensi*

A/N1S	/2	*H. saturnus* *H. suaveolens*
A/N1SM	/1	*T. globosa*
	/2 or 2B	*H. subpelliculosa*
	/2B	*C. utilis*
MA/O4	/1	*P. robertsii*
	/2A	*P. robertsii*
	/2B	*S. uvarum* (ferments galactose weakly) *S. veronae*
	/FF	*Schizosaccharomyces pombe*
MA/O1SM	/1	*S. oviformis* *S. fermentati* *T. colliculosa*
	/2	*S. oviformis*
	/2 or 2B	*S. pastorianus*
	/2B	*S. bayanus*
MA/N4	/2	*H. subpelliculosa*
MA/N1SM	/2	*H. anomala*
MB/O1SM	/FF	*Schizosaccharomyces versatilis*
B/O3	/1	*S. microellipsodes*
B/O4	/2	*C. melibiosi*
GM/O4	/1	*S. italicus* (*T. sake*, sucrose fermented weakly)
	/2B	*S. italicus* *C. claussenii* *C. albicans*
GM/N2M /N2ML		*T. anomala* (ferments lactose weakly)

GA/O3	/1	*S. exiguus* *T. holmii*
	/2	*S. chevalieri*
	/2B	*S. marxianus* *C. macedoniensis*
GA/O3L	/2B	*S. marxianus* *C. macedoniensis*
GA/O4	/2	*P. ohmeri*
	/2 or 2B	*P. guilliermondii* *C. guilliermondii*
	/2B	*S. fructuum*
GB/O3	/1	*S. microellipsodes*
GB/O4	/2B	(*S. uvarum*, ferments maltose weakly)
	/2	*C. melibiosi*
GMS/O4	/2 or 2B	*C. tropicalis*
	/2A	*T. sake*
	/2B	*S. steineri*
GMS/N4	/1	*T. versatilis*
	/2	*H. scheggii*
GMS/N5	/1	*T. versatilis*
GMA/O4	/1	*S. cerevisiae*
	/2	*S. cerevisiae* *C. robusta* *S. cerevisiae* var *ellipsoideus* *S. willianus* *Trichosporon behrendii* (large polymorphic cells with some true mycelium)
	/FF	*Schizosaccharomyces pombe*
GMA/O1SM	/FF	*Schizosaccharomyces pombe*

GMA/O5	/2	*C. intermedia*
GMA/N4	/1	*T. versatilis*
	/2	*H. anomala* *C. pelliculosa*
GMA/N5	/1	*T. versatilis*
GMB/O4	/1	*S. carlsbergensis*
	/2	*S. florentinus* *S. carlsbergensis* *S. uvarum* *S. logos*
	/FF	*Schizosaccharomyces versatilis*
GL/O3L	/2	*B. anomalus*
GML/N2M /N2ML	/1	*T. anomala*
GAL/O5	/2A	*S. lactis* *T. sphaerica*
GAL/O3L	/2B	*S. fragilis* *C. pseudotropicalis*
GMAL/N5	/1	*B. claussenii*

The primary grouping is based on the ability of a yeast to ferment 6 sugars, melibiose being used merely to determine the extent of the raffinose fermentation. Gas from raffinose alone means that only one-third of the molecule has been fermented; gas from both sugars indicates either two-thirds or all the molecule has been attacked. Theoretically, with 6 sugars there are 2^6 or 64 possible combinations, but the first Kluyver–Dekker law (Kluyver, 1931) states that if a yeast ferments any sugar, it will ferment glucose. Thus the number of combinations is reduced to 33 (including complete inability to ferment). Of these, 8 combinations include both maltose and lactose; in theory impossible, but in practice 3 yeasts ferment both of them in 2 different combinations of sugars. In Table 6, code letters are assigned to the 17 groups so far found. Unlike the code of Schultz and Atkin (1947), these letters refer not to single sugars but to several.

The second major grouping, based on the assimilation of nitrate, allows of only 2 possibilities.

The third grouping is based on the assimilation of 5 sugars, raffinose having to be omitted for lack of published information. All yeasts must assimilate glucose, irrespective of any other sugar, so that the number of groupings theoretically possible is 16, all of which are found in nature.

The total number of combinations based on these 3 groupings should be $33 \times 2 \times 16 = 528 \times 2 = 1056$. However, many of these combinations are impossible, since a sugar may be oxidatively assimilated but not fermented (Kluyver and Custers, 1939/40). The number of permissible groupings can be shown to be 99 instead of the theoretical 528, giving a true total of 99×2, or 198. The approximate number of yeast species recognized up to 1965 is 450 (Lodder and Kreger-van Rij, 1952; Soyugenc and Hedrick, 1966). Hence, some further separation of the species is desirable: using the 9 morphological characters given in Table 6, a total of 1782 becomes possible. Even so, a number of yeasts appear in Table 6 with the same coding characters. Probably one would not get such yeasts together in any one environment, but they could be differentiated by using assimilation of the much wider range of carbon compounds, proposed originally by Wickerham and Burton (1948). Thirty compounds, other than the sugars used in the fermentation tests, would yield 2^{30} or 1074×10^6 different combinations, without the problem of impossible interactions described above. The code could be made more manageable by using only 28 divided into 7 groups of 4 compounds, which could be described by 7 letters each numbered from 1 to 16. Anyone needing to differentiate between yeasts in this manner may need to subject both the unknown yeasts and possible type species to these tests, as such information is available only for recently published species. Alternatively yeast strains of industrial importance can be characterized at this point on type of flocculence, alcohol tolerance, etc., since for this purpose binomial descriptions are inadequate.

With either the Biochemical or Morphological Keys given above, it should be possible to identify any yeast species or strain and to give it a code letter that would be descriptive of its properties.

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The Determination of Deoxyribonucleic Acid Base Compositions and its Application to Bacterial Taxonomy

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The techniques of molecular biology are, at present, outside the possibilities of a routine diagnostic laboratory and are not therefore applicable to the identification of fresh isolates. None the less, the study of bacterial deoxyribonucleic acids (DNA) already assists identification by refining bacterial taxonomy. In the future, techniques may become simplified to an extent that will permit their use by the diagnostician.

Experimental Methods

Preparation of bacterial DNA

The standard procedure is that of Marmur (1961), the main steps of which are illustrated in Fig. 1. About 2–3 g wet weight of bacterial cells, suspended in 25 ml of 0·15 M NaCl + 0·1 M EDTA solution at pH 8·0, are required.

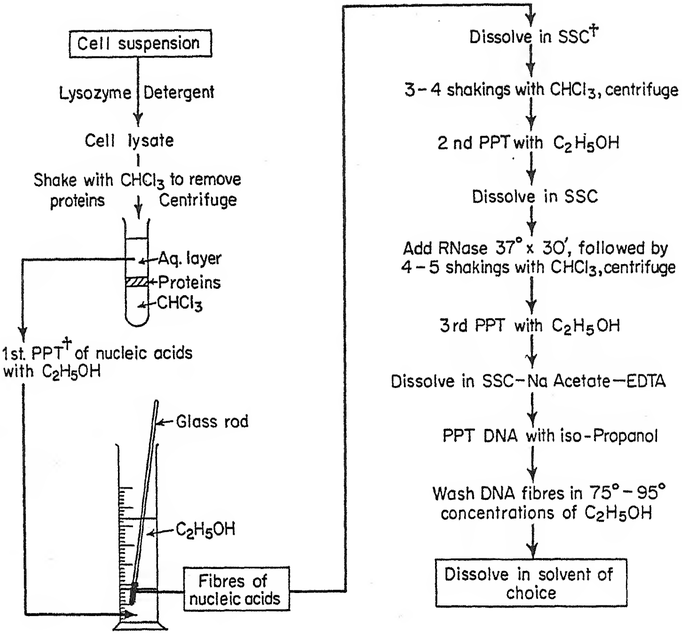
Lysis of the cells

(a) *Gram-negative and a few Gram-positive bacteria.* Heat the cell suspension with 2% sodium lauryl sulphate, a detergent, for 15 min at 60°. Lysis is observed by a clearing and by an increase in the viscosity of the suspension.

(b) *Most Gram-positive bacteria.* Collect the cells in the logarithmic phase of growth. Incubate them with lysozyme (10mg/25ml of bacterial suspension) at 37° until an increase in viscosity occurs, and then treat as (a) above.

(c) *Lysozyme and detergent resistant cells.* Penicillin (Silvestri and Hill, 1965) may be added to the growth medium, and the collected cells treated as in (a).

(d) *Physical disruption.* Cells which are not lysed by the above methods may be physically disrupted by grinding, bacterial presses, sonic disintegration, etc. However, DNA of low molecular weight usually results.



†Abbreviations: SSC = 0·15 M NaCl + 0·015 M Na_3 citrate
PPT = Precipitation

Notes: (1) 2–3 g wet weight of logarithmic phase cells required. Yield of pure DNA very variable; 1–4 mg is a good yield

(2) 6–8 hrs from cell lysate to pure DNA fibres

(3) Reference: Marmur, J. (1961). J.molec. Biol. **3** 208.

FIG. 1. DNA preparation.

Sodium perchlorate is added to the lysate to give a final concentration of 1 M in order to dissociate nucleoproteins from DNA.

Precipitation of nucleic acids

(a) *Ethanol precipitation.* Layer two volumes of 95% ethanol on top of the solution containing the nucleic acids, and mix the two layers by stirring with a glass rod inserted to just below the interface. Nucleic acids precipitate as fibres which wind on to the rod.

(b) iso-*Propanol precipitation.* Fibres previously precipitated by ethanol, as in (a), are dissolved in 9 ml of dilute standard saline citrate (SSC: sodium chloride 0·15 M, trisodium citrate 0·015 M diluted with water to one-tenth its standard concentration) and 1 ml of a solution of 3 M sodium acetate + 0·001 M EDTA. 5·6–6·0 ml of *iso*-propanol are added drop by drop to

the DNA solution, stirring continuously. As the fibres precipitate, they wind on to the rod.

Deproteinization

(a) *Chloroform*. Crude nucleic acids precipitated by ethanol as above are redissolved in standard saline citrate (SSC) and shaken in a bottle with an equal volume of a 24:1 (v/v) chloroform: *iso*-amyl alcohol mixture. This is repeated several times until no proteins appear at the water–chloroform interface after centrifugation. The upper layer is removed to a cylinder and the partially purified nucleic acids are precipitated with ethanol. The nucleic acids are again dissolved in SSC, ribonuclease (RNase) is added to a final concentration of 50 μg/ml and the solution is incubated at 37° for 30 min. It is then subjected to a second cycle of chloroform deproteinizations.

(b) *Phenol*. For the determination of base composition by spectral analysis (*vide infra*), very highly purified DNA is required. After RNase treatment, phenol may substitute chloroform as the deproteinizing agent. Again, after several shakings with cold, SSC-saturated phenol, no proteins precipitate at the water–phenol interface. Phenol dissolved in the aqueous layer is removed by several shakings with ether, and the ether is finally removed by bubbling nitrogen gas through it.

Handling of DNA

High molecular weight DNA in solution is very sensitive to shearing forces. DNA solutions are therefore poured wherever possible. When pipetting is unavoidable, wide-bore pipettes are used.

Determination of base composition

Base composition is conveniently expressed as a percentage of guanine and cytosine to total bases (% GC). Base ratios in bacterial DNA vary from species to species within a range of 25–75% GC. Methods of determining % GC are (1) from chemical analysis, (2) from the denaturation temperature, (3) from the buoyant density, (4) from spectral analysis.

1. *Chemical analysis*

DNA is hydrolysed and subjected to paper chromatography to separate and estimate the four bases. This method, largely superseded by the physioco-chemical methods below, is reviewed by Bendich (1957).

2. *Denaturation temperature*

Heating DNA in solution leads to the break-down of hydrogen bonds between bases on the complementary strands permitting the latter to

uncoil and separate. The double-stranded helix (native configuration) changes to single-stranded DNA (denatured configuration). Denatured DNA absorbs approximately 40% more ultraviolet light at wavelength (λ) 260 nm than native DNA. The temperature at which denaturation occurs can be determined by plotting optical density (O.D.) as a function of

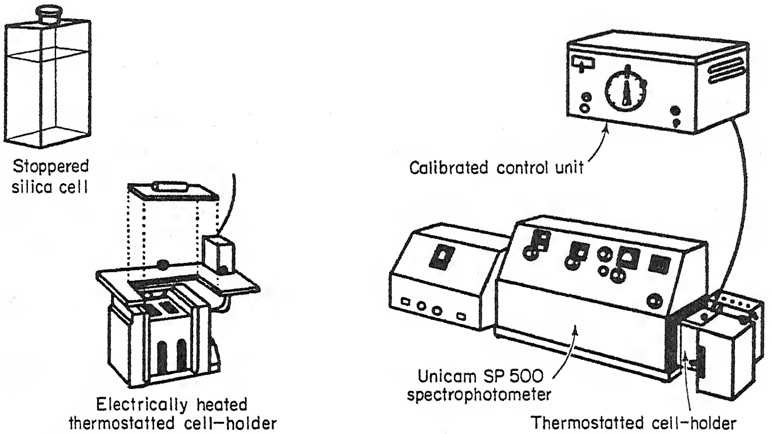


FIG. 2. Determination of %GC from heat-denaturation curves: apparatus.

temperature. The optical density initially remains constant, or decreases slightly, until the onset of denaturation, when there is a sharp increase, after which the O.D. becomes constant again. The increase in O.D. is called the hyperchromicity and the denaturation (or "melting") temperature (*Tm*) is defined as that temperature corresponding to 50% of the hyperchromicity. This determination is made with stoppered silica cells to avoid evaporation, a cell-holder fitted with a thermostatically controlled heater and a spectrophotometer. The system in use at Colindale, using an electrically heated cell-holder, is illustrated in Fig. 2. The DNA is dissolved in SSC, for example, at a concentration of 10–20 μg/ml, and is placed in a stoppered cell. A similar cell containing only solvent serves as blank. The optical density is read and the heating is switched on. When the cell reaches a pre-set temperature, the O.D. is again read and the temperature further increased. In the denaturation region, the temperature is increased only a degree or two at a time. Two curves are illustrated in Fig. 3.

The denaturation temperature is dependent on two factors, the base composition of the DNA and the ionic strength of the solvent in which the DNA is dissolved.

(a) *DNA base composition.* For any one solvent, when the ionic strength is constant, the greater the GC content the higher the *Tm*. The linear

relationship between *Tm* in SSC and % GC, derived by Marmur and Doty (1962), is illustrated in Fig. 4, and the corresponding equation is also given. The relationship is satisfactory over the range 30–70% GC; below or above this range some departure from linearity occurs.

(b) *Solvent ionic strength.* The denaturation temperature is linearly re-

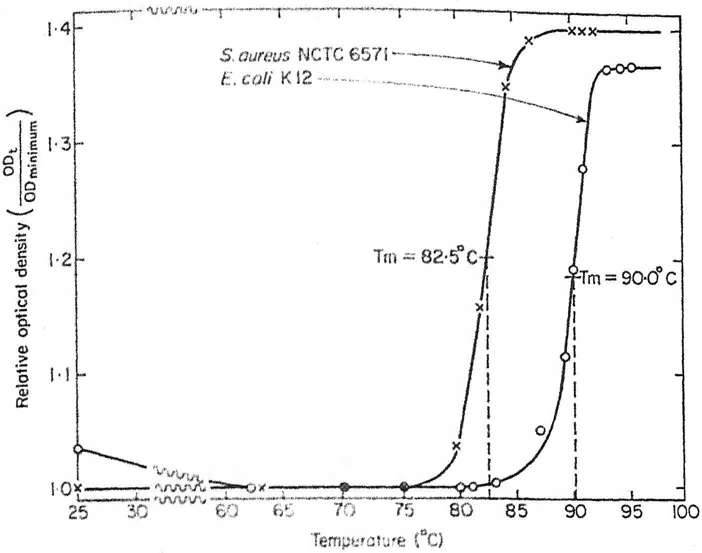


FIG. 3. Two DNA heat-denaturation curves. Temperature of DNA sample in spectrophotometer is raised slowly, optical density recorded periodically: as DNA denatures ("melts"), O.D. increases. Tm (melting temperature) = midpoint of increase in O.D. Solvent = SSC. UV light wavelength = 260 nm.

lated to the logarithm of the solvent ionic strength; the more dilute the solvent, the lower the *Tm*. Use is made of this when the DNA is of high % GC, i.e. when the *Tm* in SSC approaches 100°. A more dilute solvent is used and the expected *Tm* in SSC extrapolated from the experimental one (Silvestri and Hill, 1965). Schildkraut and Lifson (1965) have derived a single equation relating all three variables (*Tm*, % GC, solvent), in which ionic strength is expressed as molarity (M):

$$Tm = 16{\cdot}6 \log \mathrm{M} + 0{\cdot}41\,(\mathrm{GC}) + 81{\cdot}5$$

Hill (unpublished work) has derived a similar equation, expressing ionic strength as specific conductance (Sp. Cond.):

$$Tm = 17{\cdot}1 \log \text{Sp. Cond.} + 0{\cdot}41\,(\mathrm{GC}) + 100{\cdot}25$$

3. *Buoyant density*

The buoyant density of DNA increases linearly with increase in GC content.

Buoyant density is estimated by caesium chloride density gradient ultracentrifugation (Meselson, Stahl and Vinograd, 1957). The equation derived by Schildkraut, Marmur and Doty (1962), relating buoyant density (ρ) to % GC is:

$$\rho = 1{\cdot}660 + 0{\cdot}098\,(GC)$$

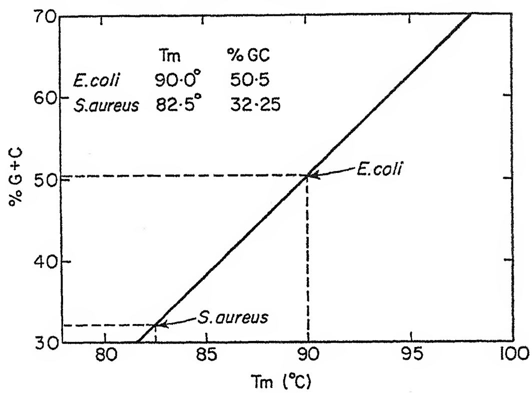


FIG. 4. Relationship between *Tm* and %GC. *Tm* is governed by two factors: (1) %GC of the DNA; (2) ionic strength of the solvent. For SSC solvent,

$$GC = (Tm - 69{\cdot}3\,/0{\cdot}41)$$

This method has the following main advantages:

(i) highly purified DNA is not required; crude nucleic acid extracts suffice;

(ii) very small quantities of DNA are used, 1–2 μg;

(iii) the linearity of the ρ to % GC relationship is maintained below and above the 30–70 % GC range;

(iv) presence or absence of "satellite" DNA differing in base composition from the main population of molecules is readily observed (Joshi, Guild and Handler, 1963; Mandel, 1966).

4. *Spectral analysis*

The UV absorbtion spectrum of DNA is a summation of the individual spectra of the four bases. A simple method of calculating % GC from absorbtion spectra of acid-denatured DNA was given by Frédericq, Oth and Fontaine (1961). A more complex method for native or heat-denatured DNA will be found in Hirschman and Felsenfeld (1966). For these methods highly purified DNA is required, as traces of proteins, whose UV absorbtion maximum is at λ = 280 nm, lead to erroneous results. Another spectroscopic method for the determination of % GC depends on the reactivity of adenine towards brominating agents (Wang and Hashagen, 1964).

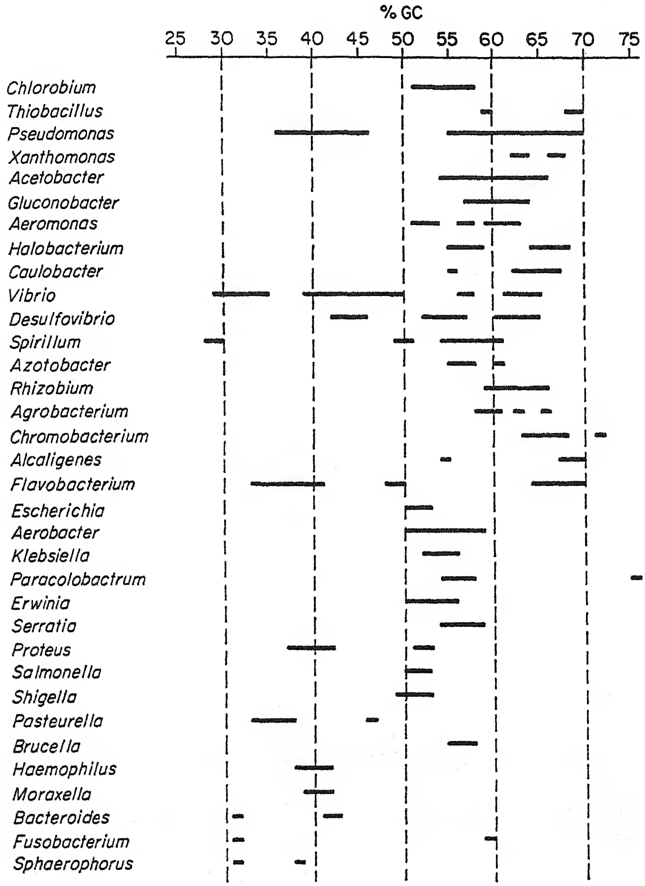


FIG. 5. Ranges of %GC values among Gram-negative genera.

Applications in Bacterial Taxonomy

Ranges of DNA base composition among bacterial genera

Base compositions of bacterial DNA are illustrated in Figs 5 and 6, taken from a recent compilation of data from the literature (Hill, 1966). The genera are arranged following mainly the classification in Bergey's Manual (Breed *et al.*, 1957). The figures show that whereas some genera are homogenous in DNA base compositions others are very heterogeneous or even discontinuous. The latter finding is more useful taxonomically than the former, for DNA samples showing widely different base composition can be assumed to have also different base sequences (i.e. genetic messages), and

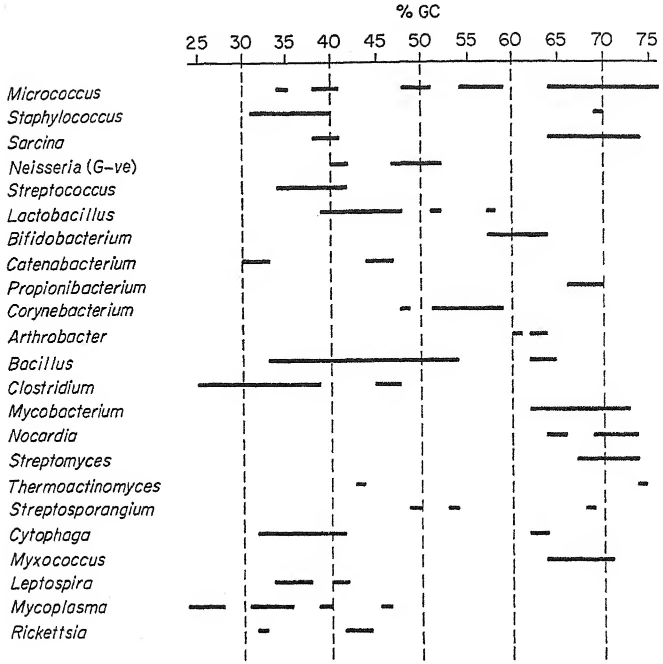


FIG. 6. Ranges of %GC values among Gram-positive and some other genera.

hence the bacteria concerned are unrelated. On the other hand, it cannot be assumed that base sequences are similar in two DNA samples because the overall base compositions are similar. Similar GC contents indicate only that the base sequences could be similar. Therefore, in those cases of genera showing homogeneity in DNA base composition, DNA data are not contradictory to the classification derived by other means. In those cases where there is heterogeneity in base compositions, within a genus, the DNA data confirms the suspicion, often already aroused by standard taxonomic methods, that unrelated bacteria are being classified together. The classification of genera can be redefined so that only organisms homogeneous with respect to base compositions are included in the same taxon.

Agreement between numerical taxonomy and DNA base compositions

In several studies there has been good agreement between taxonomic conclusions based on numerical taxonomy and those based on GC contents of DNA. An example is illustrated in Fig. 7. The *Micrococcus-Staphylococcus* group had been divided into two major taxa on numerical taxonomy evidence (Hill, 1959; Hill *et al.*, 1965), and subsequent determination of

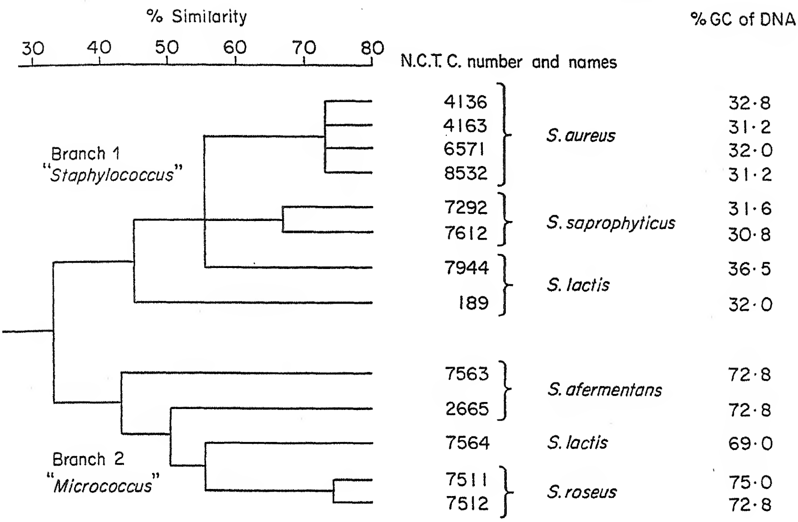


FIG. 7. Example of agreement between numerical taxonomy and DNA %GC. *Micrococcus–Staphylococcus* group divided into two major groups by numerical taxonomy (see dendrogram with % similarity scale) was confirmed by large differences in DNA base compositions.

DNA base compositions of representative strains in each taxon showed a wide difference between the two, each alone being homogeneous (Silvestri and Hill, 1965). One strain of *S. lactis* (NCTC 7564) was classified with *Micrococcus*, although the other *S. lactis* strains were more similar to *Staphylococcus*; the high GC content of the DNA of this strain confirmed its grouping with the micrococci.

Conclusions

DNA base compositions represent the first stage in the application of molecular biological methods to bacterial taxonomy. These methods are most useful when different base ratios are found in bacteria previously classified together or when used on conjunction with numerical taxonomy. The second stage of development in this field, as yet little exploited, lies in the demonstration not only of similar base compositions but also of similar base sequences. An indication of degrees of similarity in base sequences can be obtained from molecular hybridization experiments which assay the amount of reuniting that can take place between single strands of one DNA and single strands of the DNA of a different bacterium (McCarthy and Bolton, 1963).

Note

The apparatus shown in Figure 2 has since been supplemented with a commercially available thermistor thermometer, the probe of which was made to our specifications. This permits temperatures to be read directly in the DNA solution during an experiment, thus avoiding reliance upon previous calibrations of the heater unit. Instrument accuracy is $\pm 0{\cdot}25°$ from 20° to 70°, and $\pm 0{\cdot}1°$ from 70° to 110°.

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Immunofluorescence—A Useful Technique for Microbial Identification

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The technique of immunofluorescence, also known as the fluorescent antibody (or FA) technique, dates effectively from the work of Coons and colleagues (Coons, Creech and Jones, 1941; Coons *et al.*, 1942). However, it is only in the last ten years or so that FA techniques have found widespread application in bacteriology. This increase in interest grew hand in hand with the development and marketing of high-grade commercial fluorochrome reagents and fluorescent conjugates, and the growing availability of good fluorescence microscopes.

The general application of FA techniques, particularly to clinical diagnosis and medical research, can best be studied in the text of Nairn (1964), and the reviews of Beutner (1961) and Cherry and Moody (1965). The present paper describes some of the technique manipulations we have found useful during several years experience with immunofluorescence, and also highlights some recent microbiological applications of the FA technique.

Direct and Indirect FA Techniques

Fluorescent staining is usually carried out either by the so-called direct technique or by the indirect or "sandwich" technique.

For the direct technique, fluorescent conjugates are prepared from each specific antiserum under investigation.

In the indirect technique the primary specific antisera are not conjugated. Instead, a fluorescent conjugate is used which is specific for the globulins of the *animal* used for the primary serum preparation. Where the primary sera are produced in rabbits, the fluorescent conjugate is prepared from an anti-rabbit globulin antiserum produced in some other animal. High-quality commercial goat anti-rabbit fluorescein conjugate is now available (e.g. from Difco Laboratories), and conjugates against the globulins of other animal species are also on the market.

The indirect technique will often give brighter staining than the direct,

and can also be useful when the titre of the primary specific serum is too low for conjugation and use in the direct technique. However, the indirect technique takes longer to perform, and in our experience does not give as clean a microscopic picture as the direct technique.

In many bacteriological investigations it is advisable to carry out trials with the indirect technique, using a fluorescent goat anti-rabbit serum together with rabbit anti-bacteria antisera (which may be either laboratory prepared or commercial.) If the staining system is found to be specific, then it can readily be converted to the direct technique by the preparation of fluorescent conjugates from the rabbit antisera.

Although only the direct technique is detailed here, most of the manipulations discussed also apply to the indirect technique.

Materials and Preparation

Antisera

Antisera intended for the FA technique must be produced with as high a specific titre as possible. During conjugation and storage some of the specific activity is lost. A high-titre conjugate will also be more economic, as it can be diluted for day-to-day use. Dilution can also be a simple way to reduce non-specific stainings. Antisera for conjugation are prepared as for ordinary serological purposes, usually in rabbits. For bacteriological applications the injected antigen often consists of heat-killed or formalin-treated cell suspensions. Techniques for antiserum production are described in various texts, e.g. Boyd (1956).

It is advisable to obtain a sample of serum from each rabbit before injections commence. These samples can be useful for reference purposes if non-specific staining is encountered later in the investigation. If antisera are to be stored for long periods before conjugation, they are best held deep frozen, at about $-20°$.

Fluorochromes

At the present time fluorescein isothiocyanate (FITC) is probably the most commonly used label for fluorescent conjugates. In stained preparations this dye emits a brilliant apple-green fluorescence when excited by blue/ultraviolet light. High-quality FITC may be expensive (about £90 per g), but this is not usually prohibitive as only 1–3 mg FITC are required for each ml of serum. The reagent can be bought in 100 mg batches (e.g. from Baltimore Biological Laboratories, Baltimore, Maryland, U.S.A.).

In special applications a contrasting colour may be useful, and labels derived from Rhodamine B (e.g. a sulphonyl chloride derivative prepared with the lissamine rhodamine of G. Gurr Ltd, London), can be employed for

this purpose as described by Nairn (1964). However, rhodamine conjugates give much less intense fluorescence than FITC conjugates, and are not recommended for general use.

Conjugation procedures

Conjugation procedures are well described in Nairn (1964). For fluorescein conjugates, the FITC powder is best suspended in a little of the carbonate buffer. This suspension is then added drop by drop to the reaction mixture, over a period of 15–20 min. The mixture should be stirred vigorously with a mechanical stirrer while the FITC suspension is being introduced. A tiny drop of octyl alcohol can be added to control any excessive frothing caused by the stirring. We usually continue the stirring for 1 to 2 h; the reaction tube is then covered and left overnight at room temperature (up to 22°).

Purification of conjugates

After standing overnight, the FITC conjugate mixture is passed through a G.25 Sephadex column to remove residual unconjugated FITC. The separation on the column is clearly visible, and the first coloured band coming through the Sephadex consists of the required conjugated globulins, and should be collected. In the conjugate collected from the Sephadex column the serum globulins will be dilute as compared with the original serum. This is due to the carbonate buffer added to the reaction mixture, and to the phosphate-buffered saline picked up in the column. If desired, the conjugate can be brought back to the original serum volume by dialysis against a 50% buffered saline solution of polyethylene glycol mol. wt 20,000 (e.g. G. Gurr's Carbowax 20 m).

The phosphate-buffered saline (buffered saline) mentioned above with a pH of 7·1 is a commonly used diluent and washing medium in FA procedures. It consists of NaCl, 8·5 g; Na_2HPO_4 (anhydrous), 1·07 g; $NaH_2PO_4.2H_2O$, 0·39 g; distilled water, 1000 ml.

The final stage of purification is usually an absorption treatment with an acetone-dried tissue powder. The powder can be prepared in the laboratory or purchased (e.g. Difco guinea-pig liver powder). This absorption is non-serological and is intended to remove charged serum molecules that might otherwise cause non-specific staining.

Fluorescent conjugates store well at −20°. In our laboratory conjugates prepared 3–4 years ago are still satisfactory. Immediately after preparation conjugates should be dispensed in 0·2–0·5 ml amounts in small tubes or bottles (e.g. 3 in. × 3/8 in. test-tubes), closed with airtight stoppers and labelled. These are then stored deep frozen. When required a single tube of

conjugate is withdrawn from the deep freeze store and rapidly thawed. The thawed conjugate is kept refrigerated at 2–4° when not actually in use, and may remain usable for 4–6 weeks. In this way the main bulk of the conjugate remains frozen until required (repeated freeze/thaw cycles can damage the conjugate).

Preparation and fixing of FA preparations

Ordinary glass slides are used. They need to be as clean as possible. A simple way of cleaning is to use a moist paste of "Vim" scouring powder. This is smeared on the slide, and rubbed vigorously over the surface with the fingers. The slide is then held by the edges, rinsed in flowing tap water, and dried by wiping with clean absorbent tissue.

For most bacteriological investigations a smear or impression is needed. This should cover a small area of the slide (e.g. 5 mm × 5 mm), so as to conserve conjugate. Before or immediately after preparing the smear it is advisable to mark the underneath of the slide with a diamond pencil to show the approximate outline of the smear. The smear may not be visible to the eye when covered with mountant and cover slip, and the underside marking will help to locate the smear when placing the slide on the stage.

Fixing can be carried out with heat (as for some other micro-preparations), 10% formol saline (1 part 40% formaldehyde solutiob plus 9 parts phosphate buffered saline), or alcohol. The choice of fixative depends on the antigen under investigation, and the type of material in which the bacteria are suspended. We have used the formol saline (15–20 min immersion) for much of our work with salmonella and clostridial cell and flagellar antigens.

For high-quality fluorescent staining of salmonella flagella (see Fig. 2) we have used formolized suspensions to prepare the smear. When dry, the smears were fixed with Kirkpatrick's fixative, (a mixture of absolute ethanol 60 ml, chloroform 30 ml, formaldehyde (40%) solution 10 ml). The critical step is the preparation of the smear, which is obtained by gently "rolling" a drop of suspension along the length of an inclined slide (previously cleaning with scouring powder as described). When dry, all but the *first* cm or so of the smear is wiped off with a damp tissue, so that the smear does not require excessive conjugate cover.

All preparations must be well fixed, otherwise they may float off during the fairly prolonged conjugate treatment and washing. This is more likely to happen when the culture contains a high concentration of soluble nutrients, which can interfere with fixation. For this reason it often helps to centrifuge bacteria out of their growth medium. The sediment is then resuspended in distilled water and used for preparing the smear. Cultures

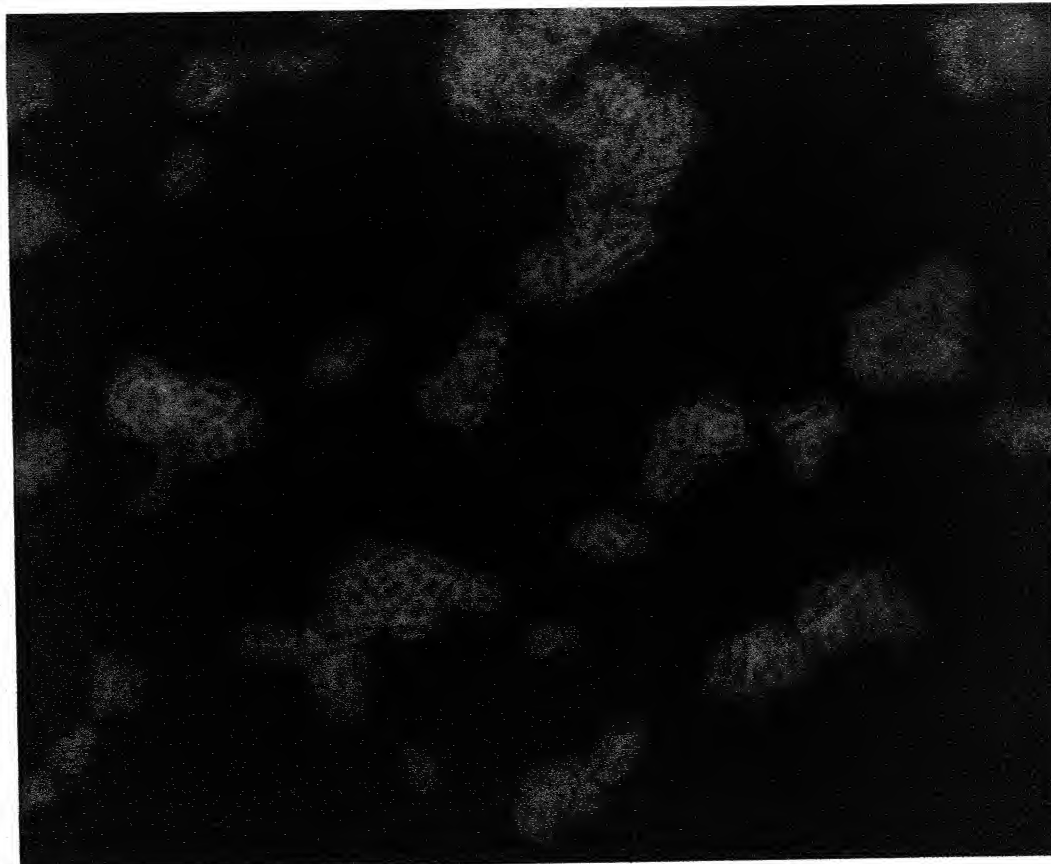

FIG. 1. Cells of *Salmonella typhimurium* stained with a FITC conjugated anti-0/4 antiserum. × 4200.

[*facing p. 190*]

FIG. 2. Cells and flagella of *Salmonella typhimurium* stained with a FITC conjugated anti-0/4 anti-H/i antiserum. × 4200.

[*facing p. 191*]

on solid media can be used to prepare smears by mixing with a drop of distilled water directly on the slide.

After fixing in formol saline or other chemical fixative, slides must be well washed in buffered saline, and then dried by blotting or by passing through alcohol and xylene baths.

Staining and mounting of smears

The dry fixed slides are placed in a container with lid (e.g. petri dish), together with moist absorbent paper. This ensures that the conjugate will not dry out when held on the slide for 15–30 min.

The conjugate is applied to the smear with a small loop. The drop of conjugate should be gently spread over the smear so as to cover the whole smear, including the outer edge. The conjugate may sometimes tend to spread further across the slide—this can be stopped by a circle drawn with a wax pencil around the outer edge of the smear.

Staining usually takes at least 15–30 min, and may take longer depending on the strength of the conjugate, thickness of smear, etc.

After staining, the excess conjugate is flushed off with buffered saline, and the slide is then immersed for 15–20 min in a dish of fresh buffered saline. Gentle agitation of the buffered saline speeds the washing process.

The washed stained slide is dried by very gentle blotting with absorbent tissue. If many slides are being handled in a slide rack, it is more convenient to dry by passing the rack through two alcohol baths, and then through a 50:50 alcohol/xylene bath and then a xylene bath. The slides are then air dried in the rack. This treatment does not cause any loss of brightness.

Fluorescein-stained slides are mounted in buffered glycerol (9 parts glycerol + 1 part buffered saline), as brightness is dependent on the pH of the surrounding medium. Lissamine Rhodamine RB200 slides can also be mounted in this way, but as RB200 is less pH-dependent, other mountants can also be used (e.g. DPX).

Glycerol-mounted preparations are not readily converted to permanent mounts. However, many preparations will maintain brightness for long periods if the cover slip is sealed with black microscope cement (G. Gurr Ltd., London) and the slides are stored deep frozen.

Microscopy

Microbiological applications of FA techniques can require high-grade optical equipment, because much work may be done at maximum useful magnifications. For the same reason, intense exciting illumination is necessary. This can readily be obtained with systems using a high-pressure

mercury lamp—the Osram HB0200 lamp is now used in several Continental and British microscopes.

Brightest fluorescence is obtained when this lamp is used with blue glass exciter filters such as the B.G. range of Schott and Genossen (Germany), together with a suitable blue and UV stopping orange barrier filter. The specimen may be illuminated with a dark-ground condenser using a thin B.G. exciter filter (2–3 mm) or by bright-field illumination using a thicker (4–6 mm) B.G. filter.

Clearest colour contrast and image quality is usually obtained with dark-ground illumination, although total brightness of fluorescence is greater with bright-field illumination.

For high magnification observation, dark-ground or high N.A. bright-field condensers should be oiled to the undersurface of the slide for maximum illumination. Liquid medicinal paraffin (mineral oil) is very useful for this purpose, as it does not fluoresce, nor does it harm the stage mechanism.

Ordinary achromat objectives are quite satisfactory for low and high magnification observation. However, at high powers better quality objectives (e.g. fluorites) will give improved results. If possible, all high-power immersion objectives should be purchased with fitted iris, as this simplifies back-and-forth changes from bright-field to dark-ground illumination.

Excellent results can be obtained with a glycerol immersion objective, such as the Reichert $\times 100$ N.A. 1·18 objective (with iris). Pure glycerol is used for immersion of this objective, and as the glycerol is completely non-fluorescent a very "crisp" image is produced.

Standard oil-immersion objectives can also be used. However, difficulty may be encountered with fluorescence from the immersion oil, even when using so-called non-fluorescent oils. This problem can become acute during the prolonged illumination required for photographic exposures. To reduce this photo-deterioration of the oil, the lamp aperture should always be kept to the minimum required.

Photomicrographs of fluorescent preparations require fast emulsions because of the low intensity of the fluorescent emission. For the same reason, the 35 mm format is preferred to larger film sizes, and the camera focusing system used should be movable and allow all the image beam to be directed on to the film during the exposure (some microscope cameras have a fixed focusing prism which removes a fraction of the image beam and lowers the total light available to the film).

At total magnifications of $\times$ 800, exposures may be 2–8 min on an ASA 160 colour film (e.g. High Speed Ektachrome). Photomicroscope exposure meters are usually not sensitive enough for estimating the correct exposure, and this is best done by experiment over a range of 1–10 min.

Some colour films can be given a special development to increase the

ASA rating, e.g. High Speed Ektachrome can then be used at an ASA rating of 300.

The prolonged exposure required for photographs means that vibration should be at a minimum. If possible, the microscope mercury lamp should be switched on several hours before a photographic session, so that the body of the microscope can reach a reasonable temperature equilibrium. This precaution will greatly reduce focusing drift, which can be a major problem during long exposures.

Applications of FA Techniques

FA techniques can be applied to almost any micro-organisms where agglutinating antisera are available or can be prepared. In any particular application, the use of FA techniques will be simplified if the antigenic characteristics of the micro-organisms are reasonably well understood, e.g. as with the salmonellae (Figs 1 and 2). In other cases, the application of FA techniques may involve considerable preliminary study of the antigenic nature of the micro-organisms under study.

Applications of FA techniques are not detailed here, but a selected list of classified references is included to illustrate our own and similar investigations on salmonellae and clostridia, and also some other interesting applications of FA techniques.

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